CLONING AND EXPRESSION OF THE *PLASMODIUM FALCIPARUM* METACASPASE GENE PFMCA1

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ABSTRACT

Programmed cell death (PCD) is a phenomenon commonly associated with multicellular organisms. Caspases are the main mediators of PCD, and this class of proteases are responsible for many of the morphological and physiological changes observed during PCD. However, in recent years, growing evidence has suggested that PCD is not unique to metazoans; unicellular eukaryotes such as *Saccharomyces cerevisiae*, *Trypanosoma brucei* and *Plasmodium spp.* have also demonstrated hallmarks of apoptosis such as DNA laddering and phosphatidylserine externalization. Metacaspases are distant homologues of caspases identified through iterative PSI-BLAST searches, and they possess the same critical catalytic dyad of cysteine and histidine residues as caspases. In *S. cerevisiae*, a metacaspase YCA1 has been shown to be involved in the cell death pathway. Similarly, three metacaspases have been identified in *P. falciparum*, the most debilitating malaria parasite in humans. Of these three metacaspases, PfMCA1 bears the most similarity to YCA1, in terms of size and identity. To elucidate the role that PfMCA1 plays in plasmodial cell death, PfMCA1 will be expressed in yeast cells, and its effect on yeast cell death will be studied. However, it was found that PfMCA1 is toxic to a variety of host cells, and this toxicity is most likely due to its catalytic activity, as the non-catalytic domain could be successfully expressed while the catalytic domain could not.

1. INTRODUCTION

1.1 Malaria

Malaria is one of the most prevalent human infections worldwide, with an estimated 300 million clinical cases and approximately 1 million deaths occurring annually (World Health Organization, Roll Back Malaria). Malaria is caused by obligate intracellular parasitic protozoan species of the genus *Plasmodium*, family *Plasmodiidae*, suborder *Haemosporidiidae*, order *Coccidia*. Four species are known to infect humans, namely *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Of these four species, *P. falciparum* is the most pathogenic, responsible for the majority of clinical cases and death (Suh *et al*., 2004).

1.1.1 The malaria life cycle

The malaria parasite spends its time between two hosts, an insect vector and a vertebrate host. In the case of humans, the parasites are exclusively transmitted by the anopheline mosquitoes; other mosquito species are responsible for transmitting the parasites in other animals, e.g. mosquitoes of the genus *Culex* can transmit avian malaria (Ejiri *et al*., 2008).

There are two phases of infection in the human host. The exoerythocytic stage begins with the bite of an infected anopheles mosquito. Infective sporozoites released into the bloodstream via the saliva of the mosquito travel to the liver, where they invade the hepatocytes and begin several rounds of replication. This process takes approximately a month; at the end, the sporozoites have matured into schizonts. In certain malaria species, such as *P. vivax* and *P. ovale*, infected hepatocytes may enter a phase of arrested development (Krotoski *et al*., 1982). The dormant hypnozoite may then remain this way for weeks to years, before it becomes active again and resumes schizogony. This delay in infection can result in clinical relapses of malaria. However, recent cases have documented that recrudescence can occur with clinical cases of *P. falciparum* infection (Foca *et al*., 2009; Greenwood *et al.*,

2008; Szmitko *et al*., 2009; Theunissen *et al*., 2009), and in *in vitro* studies (Thapar *et al*., 2005), which would pose problems for current ongoing efforts to control and eradicate the disease.

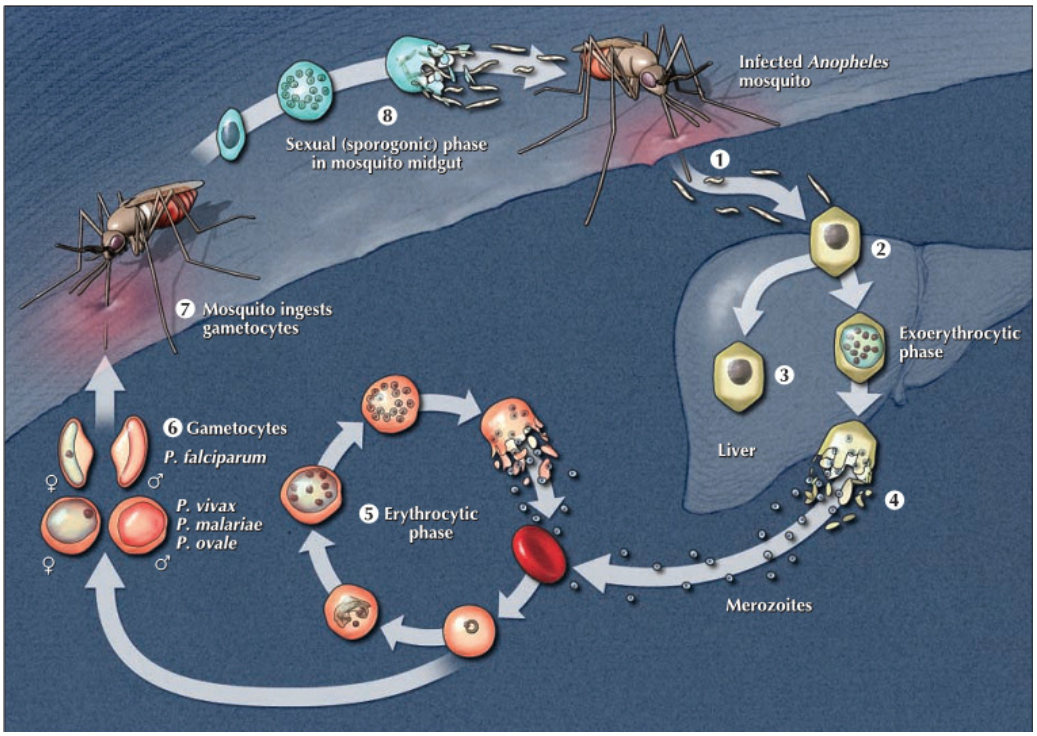

Figure 1. Life cycle of the *Plasmodium* parasite. Adapted from Suh *et al.*, 2004.

The mature schizont can contain 30,000 to 50,000 merozoites, and upon rupture of the hepatocyte, these merozoites are released into the bloodstream. The majority of the merozoites are ingested by Kupffer cells in the liver , but those that escape will rapidly invade red blood cells (erythrocytes), thus beginning the erythrocytic phase. The merozoite does not come into direct contact with the cytoplasm of the erythrocyte. Rather, it forms a parasitophorous vacuole (PV), where it will continue further development and maturation.

In the PV, the merozoite will begin differentiating into a trophozoite, breaking down erythrocytic cytoplasmic components and using them as nutrients. The trophozoites will subsequently further mature into numerous merozoites, upon which the infected erythrocyte will rupture and release the merozoites into the bloodstream, thereby repeating the

erythrocytic phase all over again. Such a cycle may take place several times in the human host.

In addition to releasing the merozoites, the rupture of the erythrocyte will also release cellular debris. This cellular debris is toxic to the host, and in synchronous infection with high enough parasitemia, this results in a significant release of cytokines by the host, and is clinically manifested as fevers. The duration of the erythrocytic stages varies between species, resulting in the fevers being of tertian or quartan periodicity.

Of the four *Plasmodium* species infecting humans, *P. falciparum* is the most life-threatening, and is almost responsible for the reported deaths attributed to malaria. There are several clinical symptoms associated with severe malaria caused by *P. falciparum*, e.g. cerebral malaria (coma), metabolic acidosis, hypoglycaemia and severe anaemia. Infected erythrocytes display several modifications to their plasma membrane, the most notable being members of the *P. falciparum* Erythrocyte Membrane Protein-1 (PfEMP1) family. PfEMP1 proteins are expressed on knob-like structures on the surface of the infected erythrocyte, and are responsible for binding to several different host vascular adhesins, such as CD36 and ICAM1. PfEMP1 also mediates binding of the infected erythrocyte to neighbouring uninfected erythrocytes, forming rosette structures. Rosetting has been hypothesized to increase the chances of a successful invasion of erythrocytes by merozoites. These properties allow the infected erythrocyte to sequester itself in the peripheral circulation and avoid splenic clearance (Kirchgatter and Del Portillo, 2005). Often, due to sequestration of such rosette structures in the vasculature, blood flow tends to be greatly decreased; binding of infected erythrocytes also causes a localised immune reaction, resulting in the release of cytokines and other mediators. This is particularly significant when it occurs in the cerebral vasculature (and is unique to *P. falciparum* infection), and can result in cerebral edema and permeabilization of the blood-brain barrier. This clinically manifests as cerebral malaria, and is a fatal complication of falciparum malaria (Warell and Gilles, *Essential Malariology*, 2002).

Upon erythrocytic invasion, a small fraction of the merozoites may not develop into trophozoites. Instead, they develop into non-multiplying sexual forms called gametocytes. These gametocytes are involved in the perpetuation of the life cycle of the parasite. When they are ingested by a feeding mosquito, they will reproduce sexually in the mosquito midgut, resulting in the production of sporozoites. These sporozoites will then travel to the salivary glands, where they will begin the entire life cycle anew (Suh *et al*., 2004; Warell and Gilles, *Essential Malariology*, 2002).

1.1.2 The burden of malaria

Approximately 90% of worldwide malaria deaths occur in sub-Saharan Africa, with the majority of these deaths being children under five years of age (World Health Organization, Africa Malaria Report 2003). The impact of malaria is mostly seen in children (Marsh *et al*., 1995), as their immune system is relatively naive and immature. The pathogenesis and morbidity of malaria results in low birth weights, improper nutrition and low attendance rates in schools. Children afflicted with malaria also suffer from learning disabilities and other neurological disorders (Holding and Snow, 2001; Kihara *et al*., 2006). Rising health costs and the loss of healthy labour causes widespread poverty and a lack of development in endemic countries (Gallup and Sachs, 2001; Sachs and Malaney, 2002). Malaria-endemic countries experience a larger-than-fivefold difference in gross domestic product than non-endemic countries, as well as slower economic growth (Sachs and Malaney, 2002). Malaria is thus not just a medical disease in these countries, but a social and economic one as well.

1.1.3 Drug resistance and targets

Chloroquine was once the drug of choice for the treatment of malaria, but widespread misuse has resulted in growing resistance in the parasites, contributing to a global resurgence of malaria cases (White, 2004). To date, malaria has known resistance to all available drug classes, with the exception of artemisinins (White, 2004). Therefore, there is an urgent need

for new drugs and drug targets, before the development of artemisinin resistance. One such attractive area for chemotherapy is pathways that unique to the parasite itself (Rosenthal *et al.*, 2002).

Cysteine proteases are important in various plasmodial process, the most critical among them being haemoglobin hydrolysis, erythrocyte invasion and rupture (Rosenthal *et al.*, 2002; Rosenthal, 2004). Falcipains and SERAs are some of the types of cysteine proteases present in the parasite. Falcipains have been implicated in haemoglobin metabolism (Rosenthal, 2004), erythrocyte invasion and egress (Blackman, 2008; Greenbaum *et al*., 2002), while SERAs are involved in erythrocyte rupture (Blackman, 2008). Cysteine proteases are thus attractive potential drug targets for chemotherapeutic intervention.

1.2 Programmed cell death (PCD)

In the 1970s, studies by Horvitz and Sulston on *Caenorhabditis elegans* revealed that out of the 1090 somatic cells that comprise the nematode, 131 of those cells will invariantly die. The process by which those cells die has been termed programmed cell death (PCD).

Apoptosis is a form of PCD, with distinct morphological and bio-chemical characteristics. It is involved in a myriad of biological processes, such as embryonic development, tissue homeostasis, and the immune response (Fadeel and Orrenius, 2005; Luder *et al*., 2001). Consequently, too much or too little apoptosis can result in a variety of human diseases, which includes cancer and neuro-degenerative diseases (Bursch, 2004). Apoptosis is characterized by various changes such as externalization of phosphatidylserine, caspase activation, nucleus fragmentation, membrane blebbing, and formation of apoptotic bodies. This highly regulated process allows the organism to eliminate any unwanted cells without causing damage to the surrounding tissue (Bursch, 2004; Philchenkov, 2004).

In contrast, necrosis as a cell death pathway is a more “violent” process, often resulting in cellular edema and leakage (Bröker *et al*., 2005). Often, necrosis is caused by

damage to the plasma membrane (Philchenkov, 2004), and the release of cellular components often results in an inflammatory response (Bröker *et al*., 2005; Bursch, 2004). Unlike apoptosis, the cell does not play an active role in its own death. Recent evidence, however, has suggested that necrosis was not the accidental and uncontrollable process that it was once thought to be, but that it is an active and regulated process (Galluzzi and Kroemer, 2008; Henriquez *et al*., 2008; Hitomi *et al*., 2008). This phenomenon of programmed necrosis has been termed necroptosis.

	Apoptosis	Necrosis	Paraptosis
Morphology			
Nuclear fragmentation	+	−	−
Chromatin condensation	+	−	±
Apoptotic bodies	+	−	−
Cytoplasmic vacuolation	−	+	+
Mitochondrial swelling	Sometimes	+	Late
Genomic effect			
TUNEL	+	Usually −	−
Internucleosomal DNA fragmentation	+	−	−
Caspase activity			
DEVD-cleaving activity	+	−	−
Caspase-3 processing	+	−	−
PARP cleavage	+ (85-kDa fragment)	+ (50- to 62-kDa fragments)	−
Inhibition by:			
zVAD.fmk	+	−	−
BAF	+	−	−
p35	+	−	−
xiap	+	−	−
Bcl-x_L	+	Usually −	−
Actinomycin D	Sometimes	−	+
Cycloheximide	Sometimes	−	+

DEVD, Asp-Glu-Val-Asp; PARP, poly(ADP-ribose) polymerase.

Table 1. Comparison of apoptosis, necrosis and paraptosis. Adapted from Sperandio *et al.*, 2000.

In recent years, other forms of PCD have been characterized. Autophagic PCD, or type II cell death, involves the digestion of cellular components by the endogenous lysosomal pathway (Bröker *et al*., 2005). This does not necessarily trigger cell death, but it allows the cell to adapt to changes in its environment (Bursch, 2004). It also allows the cell to maintain normal cellular turnover, by degrading proteins that are too old etc., as well as to function in cellular remodelling (Bröker *et al*., 2005). The critical role of the lysosomal vacuoles distinguishes autophagic cell death from apoptosis, or type I cell death, where the lysosomes are only involved much later in the death process (Bursch *et al*., 2000). In addition to cell death, autophagy has also been implicated in lifespan regulation (Dwivedi and Ahnn, 2009).

An alternative form of cell death, paraptosis, is a non-apoptotic form of PCD, i.e. it does not display the typical characteristics of apoptosis. In addition to lacking the expected apoptotic characteristics, cells undergoing paraptosis display cytoplasmic vacuolation (Sperandio *et al*., 2000) and swelling of the mitochondria and endoplasmic reticulum (ER) (Bröker *et al*., 2005).

Pyroptosis is another kind of cell death, and its features include a significant increase in the size of the cell, rapid loss of plasma membrane integrity, and release of proinflammatory intracellular constituents (Bergsbaken *et al*., 2009). In that respect, the morphological features are practically indistinguishable from necrosis. Pyroptosis, however, also demonstrates hallmarks of apoptosis, such as DNA cleavage and dismantling of the actin cytoskeleton (Bergsbaken *et al*., 2009). The molecular mediator (caspase 1) of pyroptosis is also involved in the apoptotic pathway (Bergsbaken *et al*., 2009; Galluzzi and Kroemer, 2009; Suzuki *et al*., 2007), further blurring the lines between apoptosis, necrosis and programmed necrosis.

1.3 Molecular mediators of PCD

As described above, there are several different types of PCD. However, these types of cell death may have overlapping characteristics, and are therefore not mutually exclusive (Bröker *et al*., 2005; Lockshin and Zakeri, 2002; Zakeri *et al*., 1995). The different cell death

programs also share many common signalling pathways (Bröker *et al*., 2005), and it may be necessary to recognize that a whole continuous spectrum of types of cell death exists (Bursch, 2004; Lockshin and Zakeri, 2002). For the purpose of this thesis, apoptotic markers, such as DNA damage and externalization of phosphatidylserine on the plasma membrane, will be used to investigate cell death.

1.3.1 Metazoa

Apoptosis has been extensively studied in a variety of multicellular eukaryotic organisms (metazoans), from *C. elegans* (where it was first characterized) to humans and insects (*Drosophila*).

The first step in understanding the molecular processes involved in apoptosis came when it was discovered that the *C. elegans* ced-3 gene is a homologue of the interleukin-1β processing enzyme (ICE) in humans (Yuan *et al*., 1993); subsequent overexpression of ICE in mammalian cells induced apoptosis (Miura *et al*., 1993). ICE was later renamed caspase-1, and currently, more than ten mammalian caspases have been discovered since then (Fan *et al*., 2005; Li and Yuan, 2008; Yi and Yuan, 2009).

Caspases are so-named because of its unique mechanistic action: a critical conserved cysteine residue is required for proteolysis, and protein substrates are always cleaved after an aspartate residue. Hence, cysteine-dependent aspartate specific protease (Timmer and Salvesen, 2007). In addition, a histidine residue further upstream is required for activation of the critical cysteine residue (Degterev *et al*., 2003). These two critical residues have been termed the catalytic dyad.

Caspases belong to clan CD, family C14 of the cysteine protease superfamily (Timmer and Salvesen, 2007), and they all share several common features (Degterev *et al*., 2003). All caspases possess a conserved pentapeptide sequence at their active site, QACXG. This does not translate into substrate specificities – different caspases have different optimal substrate specificities, and can be grouped as such (Degterev *et al*., 2003; Grütter, 2000).

A

Caspase substrate specificities*.

Specificity group		P4–P1, Optimal recognition motif	Consensus
Group I	Caspase-1	WEHD	WEHD
	Caspase-4	WEHD	
	Caspase-5	WEHD	
	Caspase-13	WEHD	
Group II	Caspase-2	DEHD	DEXD
	Caspase-3	DEVD	
	Caspase-7	DEVD	
Group III	Caspase-6	VEHD	(I/V/L)EXD
	Caspase-8	LETD	
	Caspase-9	LEHD	
	Caspase-10	LEXD	

B

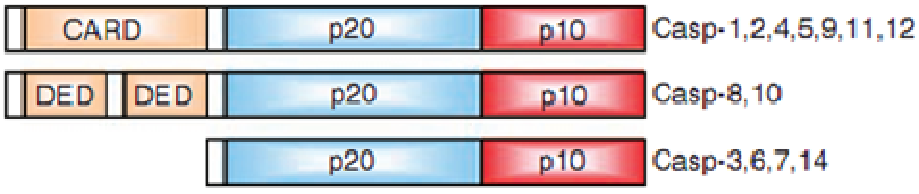

Figure 2. Grouping of caspases.
Caspases can be grouped according to **A.** their substrate specificities (Grütter, 2000) or **B.** the length of their prodomains (Li and Yuan, 2008).

All caspases are synthesized as zymogens (or procaspases), and each caspase molecule contains 4 domains: a prodomain of variable length, a p20 subunit, a p10 subunit, and a linker connecting the p20 and p10 subunits (Degterev *et al*., 2003; Philchenkov, 2004), although the linker is not present in certain caspases (Philchenkov, 2004). Activation of caspases occur when the prodomain is removed, followed by the proteolytic cleavage of the remainder protein into the two respective subunits. Two p20 and two p10 subunits will then oligomerize and form a heterotetramer, the enzymatically-active form of caspases (Fan *et al*., 2005; Grimm, *Genetics of Apoptosis*, 2003; Grütter, 2000; Philchenkov, 2004).

Caspases can be further divided into two groups based on the length of their prodomains. Caspases which possess a long prodomain are generally known as initiator caspases (Grimm, *Genetics of Apoptosis*, 2003; Li and Yuan, 2008; Philchenkov, 2004). The prodomains of caspases contain protein interaction domains, such as the caspase recruitment domain (CARD) and death effector domain (DED), which recruit the procaspases to specific complexes upon activation of upstream signals. This results in activation of the caspases via autocatalysis; activated initiator caspases can also cleave other precursors of itself in a positive feedback loop. The activated initiator caspases will then activate its downstream targets, usually the effector caspases. In certain cases, initiator caspases can also act as effector caspases, thereby amplifying the cell death signal (Philchenkov, 2004).

Effector caspases do not possess a long prodomain, and require cleavage by other proteases before they can be activated (Grimm, *Genetics of Apoptosis*, 2003). Besides initiator caspases, other non-caspase proteases such as cathepsins and calpains can also activate effector caspases (Philchenkov, 2004). Effector caspases, as the name suggests, are responsible for most of the cellular dismantling that is observed during apoptosis (Li and Yuan, 2008).

Caspases can be activated by either one of two pathways. The extrinsic pathway relies on receptors in the plasma membrane, and upon ligand binding, e.g. FAS and TNFα, the bound receptors oligomerize. This recruits adaptor proteins and procaspase, forming a protein complex, which activates the caspases. The activated caspases are subsequently

released into the cytoplasm, where they will activate downstream effector molecules, which will ultimately lead to cell death (Degterev *et al*., 2003; Fadeel and Orrenius, 2005; Fan *et al*., 2005; Grimm, *Genetics of Apoptosis*, 2003; Philchenkov, 2004).

In the intrinsic pathway, the mitochondria play a pivotal role. Under normal physiological conditions, there is a delicate balance of pro- and anti-apoptotic molecules (Grimm, *Genetics of Apoptosis*, 2003; Huang, 2002), which are members of the Bcl-2 family of proteins (Grimm, *Genetics of Apoptosis*, 2003). Depending on the stimuli received by the cell, apoptosis may be initiated or attenuated. When the cell is stressed by UV-induced DNA damage or reactive oxygen species (ROS) etc., the outer membrane of the mitochondria permeabilizes, releasing a range of proteins from the intermembrane space (Grimm, *Genetics of Apoptosis*, 2003). Proteins released include cytochrome c, apoptosis-inducing factor (AIF) and endonuclease G. The presence of cytochrome c in the cytoplasm will induce the formation of a protein complex called the apoptosome, which consists of Apaf-1, cytochrome c, dATPs and procaspase-9. Procaspase-9 is then processed into its active form, which will then proceed to activate downstream caspases (Grimm, *Genetics of Apoptosis*, 2003; Li and Yuan, 2008).

1.3.2 Protozoa (including *Plasmodium spp.*)

Apoptosis has been studied exhaustively in metazoans, as the altruistic nature of PCD suggests an obvious benefit for multicellular organisms. The idea that PCD could exist in unicellular organisms, such as bacteria and protozoan (unicellular eukaryotes), seemed illogical and counter-intuitive – there seems to be no reason at all why an individual cell would commit suicide.

However, certain unicellular eukaryotes have been observed to display features which are normally associated with apoptosis in metazoans. These organisms include *Trypanosoma* (Ameisen *et al*., 1995; Piacenza *et al*., 2001; Ridgley *et al*., 1999; Welburn *et al*., 1996), *Leishmania* (Arnoult *et al*., 2002; Das *et al*., 2001; Lee *et al*., 2002; Moreira *et al*., 1996), *Plasmodium* (Al-Olayan *et al*., 2002; Deponte and Becker, 2004; Hurd and Carter, 2004;

Hurd *et al*., 2006; Le Chat *et al*., 2007; Meslin *et al*., 2007; Picot *et al*., 1997), the slime mold *Dictyostelium discoideum* (Cornillon *et al*., 1994), the ciliate *Tetrahymena thermophila* (Christensen *et al*., 1995), the dinoflagellate *Peridinium gatunense* (Vardi *et al*., 1999), the intestinal protozoan parasite *Blastocystis* (Nasirudeen *et al*., 2001a, 2001b, 2004; Nasirudeen and Tan, 2004, 2005; Tan *et al*., 2001; Tan and Nasirudeen, 2005) and *Saccharomyces cerevisiae* (Granot *et al*., 2003; Ludovico *et al*., 2001; Madeo *et al*., 1997, 1999, 2004).

Several reasons have been postulated to explain cell death in unicellular organisms. One proposes that cell death is an altruistic response, and that certain cells, such as those which produce a large amount of reactive oxygen species, will die preferentially. This conserves limited resources, and benefits the entire population (Hurd and Carter, 2004). In parasites, cell death would also serve as a mechanism for limiting the population size, to allow for successful transmission (Al-Olayan *et al*., 2002; Das *et al*., 2001; Hurd and Carter, 2004). A lower parasite load would also limit the intensity of infection and allow for a higher host survival rate.

Although markers of apoptosis have been observed and characterized in protozoan, no molecular mediators homologous to those found in metazoans were found until recently, such as when endonuclease G was found to be involved in trypanosome cell death (Gannavaram *et al*., 2008). Indeed, the absence of caspases, which play a major and important role in metazoan apoptosis, was a great obstacle to proving that a conserved pathway exists in both metazoans and protozoan (Madeo *et al*., 2002), even though heterologous expression of Bax (a metazoan pro-apoptotic mediator) was shown to be lethal to *S. cerevisiae* (Greenhalf *et al*., 1996; Ligr *et al*., 1998; Madeo *et al*., 1999; Manon *et al*., 1997). Conversely, heterologous expression of the metazoan anti-apoptotic mediators Bcl-2 and Bcl-xL increases the survival rate of senescent yeast cells (Longo *et al*., 1997) and those which have been exposed to H_2O_2 (Chen *et al*., 2003). Overexpression of Bcl-xL also rescued yeast cells which co-overexpressed Bax, preventing the appearance of apoptotic features (Greenhalf *et al*., 1996; Ligr *et al*., 1998; Manon *et al*., 1997). Taken together with the fact that no homologs of Bax, or other members of the Bcl-2 family (Priault *et al*., 2003), have been identified in the yeast

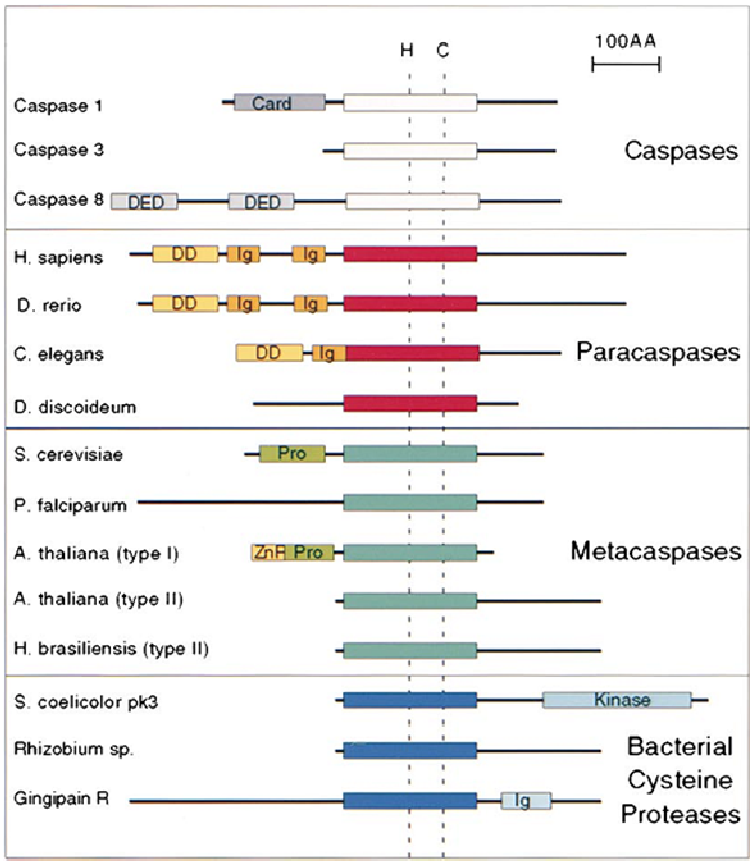

Figure 3. Domains of caspases, paracaspases and metacaspases. All possess the conserved histidine and cysteine residues required for catalytic action (Uren *et al.*, 2000)

genome, these observations suggest that the apoptotic machinery may be conserved between unicellular and multicellular eukaryotes (Greenhalf *et al.*, 1996).

In 2000, Uren *et al.* identified two families of caspase-like proteins using iterative PSI-BLAST searches (Uren *et al.*, 2000). Paracaspases are found in metazoans and *Dictyostelium*, while metacaspases are found in plants, fungi and protozoa. Alignment of the novel sequences with classical caspases showed that the conserved cysteine and histidine residues are both present in paracaspases and metacaspases.

Depending on the tertiary structure and sequence similarity, metacaspases can be divided into two classes. Type I metacaspases are generally found in plants and fungi, and they contain prodomains with a proline-rich repeat motif. In the case of plant type I metacaspases, they may also possess a zinc finger motif. Type II metacapases typically do not

possess any prodomains; however, they have a 200 residues insertion located C-terminally of their catalytic domain.

Following the discovery of metacaspases, a metacaspase YCA1 was found to be involved in yeast apoptosis. YCA1 undergoes a cleavage pattern similar to classical caspases, and is activated when yeast is exposed to apoptotic stimuli. In addition, a YCA1 knockout yeast strain increases resistance to apoptosis caused by H_2O_2 or senescence. Conversely, overexpression of YCA1 leads to increased sensitivity to apoptosis-inducing stimuli (Madeo *et al*., 2002).

In addition, metacaspases from other organisms, such as *Trypanosoma* (Szallies *et al*., 2002), *Leishmania* (González *et al*., 2007), *Candida* (Cao *et al*., 2009), the fission yeast *Schizosaccharomyces pombe* (Lim *et al*., 2007), the Norway spruce *Picea abies* (Bozhkov *et al*., 2005), and *Arabidopsis thaliana* (Watanabe and Lam, 2005), demonstrated a similar function, thus further adding weight to the idea of a conserved apoptotic pathway between protozoans and metazoans.

Despite the apparent functional similarity, metacaspases differ from traditional caspases in certain ways. Unlike caspases, which requires an aspartate residue at the substrate P1 position, initial 3D modeling showed that metacaspases prefer uncharged residues at that position (Uren *et al*., 2000). However, it appears from work done on *Arabidopsis* (Vercammen *et al*., 2004; Watanabe and Lam, 2005), *Trypanosoma* (Moss *et al*., 2007) and *Leishmania* (González *et al*., 2007; Lee *et al*.,2007) metacaspases that they prefer basic residues, namely arginine or lysine, at the P1 position. The change in amino acid preference may be a reason why metacaspases are insensitive to caspase-specific molecules, such as substrate peptides and inhibitors, but are sensitive to serine protease inhibitors (Bozhkov *et al*., 2005; Vercammen *et al*., 2004; Watanabe and Lam, 2005). Thus, while caspase-like activities have been reported in organisms possessing metacaspases (Al-Olayan *et al*., 2002; Bozhkov *et al*., 2004; Das *et al*., 2001; Hoeberichts and Woltering, 2003; Kosec *et al*., 2006; Lam and del Pozo, 2000; Lee *et al*., 2002; Madeo *et al*., 2002, 2004; Thrane *et al*., 2004), it

would seem that metacaspases are not responsible for such activities, even though they appear to be involved in the apoptotic machinery.

1.4 Objectives of study

Proteases of parasitic protozoa, particularly cysteine proteases, are attractive targets for chemotherapy, as they play key roles in various biological processes, from invasion of host cells, to pathogenesis (Mottram *et al*., 2003; Rosenthal *et al*., 2002; Rosenthal 2004; Wu *et al*., 2003). In the case of a debilitating disease such as malaria, resistance to conventional drugs are becoming increasingly more common (Rosenthal *et al*., 2002; Rosenthal 2004; Wu *et al*., 2003), and it is more necessary than ever to discover new drug targets that might aid in the control, if not eradication, of this disease.

As described above, metacaspases have been implicated in apoptosis in a variety of protozoa that lacks classical caspases. *S. cerevisiae* has traditionally been used as a model organism to study various cellular processes (Fröhlich *et al*., 2007), and the ease of manipulation and many readily-available established protocols makes the yeast model system an excellent candidate for studying *Plasmodium* metacaspases. The yeast metacaspase YCA1 has been characterized, and wild-type and YCA1-knockout strains are readily available. In *P. falciparum* itself, three putative metacaspase genes have been identified (Le Chat *et al*., 2007). A BLAST search revealed that one of them, PfMCA1 (PlasmoDB gene ID PF13_0289), bears 42% similarity to YCA1, making PfMCA1 a good candidate for studying the functional role of metacaspases in *P. falciparum* apoptosis.

The first objective of this study would be to clone the PfMCA1 gene into both wild-type and YCA1-knockout yeast. The functional effect of PfMCA1 expression, with regards to cell death, will be investigated. If PfMCA1 has a function similar to YCA1, it should increase sensitivity to cell death stimuli.

The second objective would be to engineer epitope tags into the PfMCA1 protein to allow for affinity purification. Purified PfMCA1 can be used to study its characteristics, such

as its enzyme kinetics, substrate and inhibitor specificity. Hopefully, understanding its biochemical characteristics would provide targets for drug intervention.

2. MATERIALS & METHODS

2.1 *Plasmodium falciparum*

2.1.1 Laboratory culture

In vitro culture of *P. falciparum* strain 3D7 was cultured in RPMI media supplemented with 0.5% (w/v) Albumax II (Gibco), 2 mM L-glutamine (Sigma-Aldrich), 0.005% (w/v) hypoxanthine (Sigma-Aldrich) and 10 mg/L of gentamycin (Gibco), at 37°C, and gassed with a nitrogen-balanced air mixture containing 5% O_2 and 5% CO_2. Haemotocrit was maintained at 2.5% and parasitemia was never allowed to rise beyond 15%. Culture medium was changed every two days.

To monitor the culture, a thin blood smear was prepared on a microscope glass slide. The culture flask was shaken gently to homogenise the culture, and a 100 µl aliquot was taken for the smear. The aliquot was centrifuged briefly to pellet the erythrocytes, and the supernatant was removed. The pellet was resuspended in the residual supernatant, and the resuspension was smeared onto the glass slide. The smear was allowed to air-dry, and methanol was used for fixation. The fixed smear was then treated with Giemsa stain for 15 minutes, after which any excess stain was washed off with tap water. The smear was then blot-dried, and viewed under a conventional optical microscope.

2.1.2 Isolation of genomic DNA

Genomic DNA was extracted from infected erythrocytes following a protocol from *Methods in Malaria Research* (Ljungström *et al*., 2004). Briefly, 10 ml of parasite culture (10% parasitemia) was centrifuged at 3,000g for 2 minutes. The supernatant was discarded, and the cell pellet was washed once with cold PBS. The infected erythrocytes were resuspended in 1 ml of PBS and 10 µl of 5% saponin was added. Upon observation of clarification (complete erythrocytic lysis), the mixture was centrifuged at 6,000g for 5 minutes. 25 µl of lysis buffer (40 mM Tris-HCl (pH 8.0), 80 mM EDTA, 2% SDS, 25 µg/ml proteinase K, 10 U/ml RNase) and 75 µl of distilled water was added to resuspend the pellet,

and the mixture incubated at 37°C for 3 hours. Phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma-Aldrich) was used to purify the genomic DNA.The aqueous layer was recovered, and used for another round of phenol-chloroform extraction. Any residual phenol remaining in the aqueous layer was removed by a wash step with chloroform. The genomic DNA was precipitated from the aqueous layer by adding 0.1 volume of sodium acetate and 2.5 volumes of absolute ethanol. The mixture was incubated at -20°C for an hour, before centrifugation at 2,000g for 30 minutes at 4°C. The DNA pellet was washed once with 70% ethanol, centrifuged at 2,000g for 30 minutes at 4°C, and air-dried. The dried DNA pellet was then resuspended in 50 µl of sterile deionised water.

2.1.3 Isolation of *P. falciparum* total RNA

P. falciparum strain 3D7 cultures were grown to high parasitemia (15-20%), and pure parasites were obtained via saponin lysis of erythrocytes (as described previously in section 2.1.2). 1 ml of TRIzol (Invitrogen) was added to the cell pellet and transferred to a 1.5 ml tube after homogenising. 10 µl of Triton X-100 was added to the sample, and sonication was used to lyse the parasites. 200 µl of chloroform was added, and the mixture was vortexed vigorously for 30 seconds. The mixture was then centrifuged at maximum speed for 5 minutes in a table-top microcentrifuge. The aqueous layer was transferred to a new tube and 400 µl of ice-cold isopropanol was added. The RNA was allowed to precipitate by incubating the mixture at -20°C for 2 hours. The precipitated RNA was then pelleted by centrifugation at maximum speed in a microcentrifuge for 15 minutes at 4°C. The supernatant was removed, and the RNA pellet was washed with 70% ethanol prepared with DEPC (diethylpyrocarbonate)-treated water. The washed pellet was air-dried, and the RNA was resolubilized in 50 µl of sterile DEPC-treated water (Invitrogen).

2.1.4 Quantification of *P. falciparum* total RNA

The concentration of RNA was determined spectrophotometrically using the NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies Inc.), and its associated computer program at the RNA-40 setting. 2 µl of the RNA sample was used per measurement. In addition, the ratio of the absorbance at 260 nm to the absorbance at 280 nm

was used to determine the purity of the RNA. Pure RNA has a ratio of 1.7 to 2.1 (Applied Biosystems TechNotes, *Critical Parameters for Successful RNA Amplification*), and the values obtained from samples typically fall within this range.

2.1.5 Preparation of *P. falciparum* cDNA

P. falciparum total RNA was treated with DnaseI (Promega) according to manufacturer's instructions. 100 ng of the Dnase-treated total RNA was then used for first-strand cDNA synthesis using the RevertAid^{TM} H-minus M-MuLV reverse transcriptase from Fermentas (according to the manufacturer's protocol), and oligo-dT primers. The reaction mixture was incubated for 60 minutes at 42^{o}C. 4µl of the mixture was then used for PCR.

2.1.6 PCR amplification of metacaspase gene PfMCA1

The following primers were used to amplify the PfMCA1 gene from *P. falciparum* genomic DNA: 5'PfMCA-EcoRI (GCCGAATTCATGGAAAAAATATACGTCAAAAT) and 3'PfMCA-SalI (GGGCGTCGACTAAAAAAAAAATAAATTTTTAAGTTC), with the EcoRI and SalI restriction sites underlined respectively. Subsequently, the reverse primer was modified to include a hexahistidine tag at the C-terminus of the PfMCA1 protein: 3'-PfMCA-6×His-SalI (GGCGTCGACTA**GTGATGATGGTGATGATG**AAAAAAAAATAAATT-TTTAAGTTC). The SalI restriction site is underlined, while the nucleotide sequence for the hexahistidine tag are in bold. EcoRI and SalI restriction sites were used for unidirectional cloning into the yeast shuttle plasmid vector PactTHA423.

PCR was performed using the Expand High Fidelity PCR kit (Roche) using the following conditions: initial denaturation was carried out at 95^{o}C for 1 minute; 5 cycles of denaturation at 95^{o}C for 1 minute, annealing at 51^{o}C for 1 minute, and elongation at 72^{o}C for 2 minutes; an additional 25 cycles of denaturation at 95^{o}C for 1 minute, annealing at 51^{o}C for 1 minute, and elongation at 72^{o}C for 2 minutes, with the duration for the elongation step increased by 5 seconds every cycle; elongation at 72^{o}C for 7 minutes; a final hold step at 16^{o}C.

2.1.7 Optimization of PfMCA1 for yeast expression

The coding sequence for PfMCA1 was optimized for protein expression in *S. cerevisiae* by reverse-translating the PfMCA1 protein sequence to a codon-optimized nucleotide sequence. The optimized coding sequence was synthesized by a commercial vendor (Genscript, Piscataway, NJ), and included a hexahistidine tag after the start codon.

2.1.8 PCR amplification of yeast-optimized PfMCA1

The larger-than-average size (2.3 kilo base-pairs) of *P. falciparum* genes (Gardner *et al*., 2002), and its high (A+T)-content pose significant obstacles to successful gene expression (Withers-Martinez *et al*., 1999; Yadava and Ockenhouse, 2003; Zhang *et al*., 2002). To increase the level of protein expression, a PfMCA coding sequence optimized for yeast expression was generated by incorporating a yeast codon bias and decreasing the (A+T)-content. The optimized DNA sequence of PfMCA1 was amplified by using the following primers: OpPfMCA-fw (GCCGAATTCATGCACCACCATC) and OpPfMCA-rv (TATAGCGGCCGCGAAGAAAAATAAATTC). The EcoRI and NotI restriction sites are underlined respectively. A forward primer OpPfMCA-noHis-fw (GCCGAATTCATGGAGA-AAATTTATGTCAAG) which amplifies the PfMCA1 gene without the hexahistidine tag was also used, in situations where the hexahistidine tag was not required.

PCR conditions were the same as that described above in section 2.1.6.

2.1.9 Site-directed mutagenesis of PfMCA1

The catalytic domain possesses two critical residues, a histidine at position 404, and a cysteine residue at position 460. In order to replace the critical cysteine residue with alanine, a set of primers, OpPfMCA-C460A-fw (GCTGTTGTAGATTCGGCTAATAGCGGTTCTTC) and OpPfMCA-C460A-rv (GAAGAACCGCTATTAGCCGAATCTACAACAGC) primers containing the mutation (C460A) were designed. These primers are reverse complements of each other.

The forward primer for the PfMCA1 gene (OpPfMCA-noHis-fw), was used with the reverse primer containing the mutation (OpPfMCA-C460A-rv), while the reverse primer for the PfMCA1 gene (OpPfMCA-rv) was used together with the forward primer containing the

mutation (OpPfMCA-C460A-fw), to generate two sets of PCR products. The PCR products were purified using the PCR Purification Kit (QIAgen) according to manufacturer's instructions. A second round of PCR was carried out using the purified products themselves as primers. The full-length gene containing the mutation was then purified via gel electrophoresis using a 2.0% (w/v) agarose gel (QIAgen Gel Purification Kit), and verified via DNA sequencing.

The PCR conditions used were the same as that described above for the amplification of PfMCA1 (section 2.1.6).

2.1.10 Molecular cloning and screening

After purification of the desired PCR fragments, they were digested with the appropriate restriction enzymes. The digestion reactions were carried out overnight at 37^{o}C. In order to minimize STAR activity (unspecific digestion) while ensuring most of the PCR fragments were digested, as little restriction enzyme as possible was used. Typically, 1 unit of restriction enzyme was added to a 60 µl reaction volume.

After digestion, the digested PCR products were purified with the QIAgen PCR Purification Kit. They were then ligated with the plasmid vector, which had been digested with the same restriction enzymes, using T4 DNA ligase (New England Biolabs), following manufacturer's instructions. In addition, the plasmid vector had been treated with Antarctic Phosphatase (New England Biolabs), as per manufacturer's instructions, after restriction enzyme digestion to prevent re-circularization. The ligation was carried out overnight at room temperature.

Competent *E. coli* cells were added to the ligation mix for transformation, and positive colonies were screened, as described below in section 2.2.4.

2.1.11 DNA sequencing

As PfMCA1 is a large gene (1,842 base-pairs), several sequencing primers needed to be designed in order to accurately sequence the entire gene. Sequencing was done both in the 5'→3' and 3'→5' directions, and started from the regions flanking the multiple cloning site (approximately 100-200 base-pairs upstream/downstream). However, the entire gene was not

sequenced completely in either direction. Instead, each would only sequence approximately 60% of the gene, and there would be a region of overlap in the middle portion. In addition, sequencing primers were set approximately 400 base-pairs apart, to provide some degree of continuity between consecutive primers.

DNA sequencing was carried out using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and using a modified manufacturer's protocol. Briefly, water was added to the reaction mixture containing 3 µl of the Ready Reaction Mix, 3 µl of 5× Sequencing Buffer, 3.2 pmol of primers and 2 µl of DNA template, to a total volume of 15 µl. The PCR was carried out using the following parameters: an initial denaturation cycle at 96^{o}C for 1 minute; 25 cycles of denaturation at 96^{o}C for 10 seconds, annealing at 50^{o}C for 5 seconds, and elongation at 60^{o}C for 4 minutes; and a final holding step at 16^{o}C. The thermal ramp rate was set at 1^{o}C/s.

The products were purified using the ethanol/EDTA precipitation method, as recommended by Applied Biosystems. Briefly, 5 µl of 125 mM EDTA was added to the sequencing mix, followed by 60 µl of absolute ethanol. The mixture was mixed by gentle pipetting, transferred to a 1.5 ml eppendorf tube, and incubated at room temperature for 15 minutes. After incubation, the mixture was centrifuged at 3,000g for 32 minutes at 4^{o}C. The supernatant was carefully removed, and the DNA pellet was washed with 60 µl of 70% ethanol. The mixture was further centrifuged at 2,000g for 15 minutes at 4^{o}C, and the supernatant carefully removed. The pellet was then dried at 50^{o}C in a heat block. The dry pellet was then sent for reading by the ABI PRISM® 3100 Genetic Analyzer.

Plasmid Vector	Name	DNA Sequence
PactTHA423	PfMCA-Pact-5'-2295	CCTCACCCTAACATATTTTCCAATTAAC
	PfMCA-Pact-5'-2700	CTTACTGCTTTTTTCTTCCCAAG
	PfMCA-Pact-5'-3100	ATTGATGTTGTAAAGAAATGTACATTGC
	PfMCA-Pact-5'-3500	ATAGCACTTATATGAACAATTCACCTAC
	PfMCA-Pact-3'-3800	GTACAACCATTCAATTCATATTTGG
	PfMCA-Pact-3'-4300	AAGAAACTTCCTTATCTTTACATCCAC
	PfMCA-Pact-3'-4700	AGGGTGGTTTAAAAATAGAAATAGAG
	PfMCA-Pact-3'-5025	AAAACGCCGGACTCAAATTCTAATG
Pgal1-HA-PL-Tactin-423	Pgal-5'	AAATCCACATAACTGACAAAACTGG
	PfMCA-Pgal-5'-3740	CCAAATTATAGACCTACAAGAAGAAATA
pESC-HIS	PfMCA-pESC-fw-4098	GGAGAGTCTTCCTTCGGAGG
	PfMCA-pESC-fw-4495	CATGTATCTTGCAGAAGAATCCATAC
	PfMCA-pESC-fw-4893	ATTGGACAGTATAACAATATATACTTTAACG
	PfMCA-pESC-fw-5301	CCGGGAAGTGATCAAACTTTATAC
	PfMCA-pESC-rv-5202	GATTGGAGTTATGTAAATCATTAGATGC
	PfMCA-pESC-rv-5601	GACCAGAAAATAGGAAGAACAGAATG
	PfMCA-pESC-rv-6001	GTAATAATCGAAGGAGTGTTCATATTATTC
	PfMCA-pESC-rv-6400	TATCTACCAACGATTTGACCCTTTTC

Table 2. List of sequencing primers used for the various clones of PfMCA1. The original sequence of PfMCA1 was used for the plasmid vectors PactTHA423 and Pgal1-HA-PL-Tactin-423. As the sequence used is the same, the first and last sequencing primer was changed according to the plasmid vector. The optimized PfMCA1 sequence was used for cloning into pESC-HIS. The number at the end represents the position of the primer.

2.1.12 SEG analysis of PfMCA1

Regions of low complexity are regions in the protein sequence where there is a periodic repetition of certain amino residues, and can hinder the successful expression of a gene (Birkholtz *et al*., 2008). The protein sequence of PfMCA1 was entered into an online SEG program (http://mendel.imp.ac.at/METHODS/seg.server.html) to determine the low complexity regions that are present. The parameters used were the same as that employed by Pizzi & Frontali (2001): window length: 45; trigger complexity: 3.4; extension complexity: 3.75.

2.2 *E. coli*

2.2.1 Bacterial strains and culture

E. coli strain DH5α cells were used for amplification of recombinant plasmids, and *E. coli* strain BL21 (DE3) cells were used for protein expression and purification.

All strains were grown in Luria-Bertani (LB) broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl), and in the case of transformed bacterial cells, with the presence of 100 µg/ml ampicillin. Bacterial cells were grown at 37°C, 220 rpm in a shaking incubator.

2.2.2 Plasmids

The pGEX vector plasmid (Amersham Biosciences) was used for heterologous protein expression in *E. coli*. The strain of pGEX used was pGEX-4T-1, which allowed in-frame cloning with the EcoRI restriction site at the 5'-end of the gene sequence. Protein expression can be controlled with the presence of isopropyl β-D-1-thiogalactopyranoside (IPTG) – presence of IPTG will induce protein expression.

2.2.3 Molecular cloning

Molecular cloning of desired gene fragments into the pGEX vector was carried out as described in section 2.1.10. The restriction enzyme sites used are EcoRI at the 5'-end and NotI at the 3'-end.

2.2.4 Preparation of competent *E. coli* cells

An overnight 2 ml bacterial culture was diluted in 125 ml of LB medium, and incubated at 37°C for 2 hours. The culture was then centrifuged at 2,000 rpm for 10 minutes, and the supernatant was removed. The cell pellet was kept on ice for 10 minutes, after which it was resuspended in 40 ml of CCMB medium (80 mM $CaCl_2$, 20 mM $MnCl_2$, 10 mM $MgCl_2$, 10 mM KCl, 10% glycerol (v/v), pH 6.4), and kept on ice for 20 minutes. The cell suspension was then centrifuged again at 2,000 rpm for 10 minutes, and the cell pellet was resuspended in 10 ml of fresh CCMB medium. The cell suspension was then aliquoted and flash-frozen in liquid nitrogen before being kept at -80°C.

2.2.5 Transformation and screening

40 µl of the competent cells was added to the plasmid solution. This mixture was homogenised gently, and incubated on ice for 20 minutes. The mixture was then heat-shocked at 42°C for 90 seconds, after which 100 µl of LB medium was added. This mixture was incubated at 37°C for 1 hour before it was plated on LB agar plates containing 100 µg/ml

ampicillin. As all the plasmid vectors used used ampicillin as a bacterial selection marker, agar plates used for bacterial cultivation contained ampicillin. The agar plates were incubated at 37°C overnight, and observed for colony growth the next day.

Colonies were screened using colony PCR. Picked colonies were inoculated into 1 ml of LB broth containing 100 µg/ml of ampicillin, and incubated at 37°C, with shaking at 220 rpm for 1 hour. 1 µl of the inoculated broth was added to 49 µl of PCR reaction mix containing a forward primer specific for the promotor in the plasmid vector, and a reverse primer specific for the cloned gene. This ensured that the gene was cloned correctly and is in the correct orientation. 4 ml of LB broth with ampicillin was then added to cultures which gave a positive band of the correct size, and incubated overnight. Overnight cultures were then used for plasmid isolation using the QIAprep Spin Miniprep Kit (QIAgen), following manufacturer's instructions.

2.2.6 DNA sequencing

The following pGEX sequencing primers were used to sequence the cloned gene: pGEX-fw (GGGCTGGCAAGCCACGTTTGGTG) and pGEX-rv (CCGGGAGCTGCATGT-GTCAGAGG).

Sequencing was carried out as described in section 2.1.11.

2.2.7 Induction of protein expression

1 ml of *E. coli* strain BL21 (DE3) transformed with the appropriate plasmid was grown overnight in LB broth in the presence of ampicillin at 37°C and shaking at 220 rpm. A 100 µl aliquot of the overnight culture was added to 1 ml of fresh LB broth with ampicillin. The freshly-inoculated cultures were then incubated with shaking for 3-5 hours at room temperature, before the addition of IPTG to a final concentration of 1 mM. The cultures were further incubated for an additional hour before the bacterial cells were harvested.

2.2.8 Isolation of bacterial protein extracts

Cultures were centrifuged at 1,000g for 10 minutes, and the supernatant was discarded. The cell pellet resuspended in 200 µl of ice-cold PBS buffer, and the resuspension

was sonicated. Lysis was deemed complete when the resuspension became translucent. The total cell lysate was centrifuged at 13,000 rpm, and 5 µl of the supernatant was used for SDS-PAGE.

2.2.9 Immunoblotting

The protein sample was mixed with an equal volume of Laemmli sample buffer (Bio-Rad) as per manufacturer's instructions, and boiled at 100^{o}C for 5 minutes. The mixture was then electrophorectically separated on a 12% SDS-PAGE gel running at 100V for 1 hour using the Mini-PROTEAN® 3 Cell (Bio-Rad). The gel was then equilibrated in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 15 minutes before being electrophorectically transferred to a nitrocellulose membrane at 20V for 30 minutes using the Trans-Blot® Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). The membrane blot was then blocked with PBST (PBS, 0.1%(v/v) Tween 20) solution containing 5% nonfat milk for 1 hour. After washing with PBST, the blocked membrane blot was incubated with PBST containing the primary antibody and 1% nonfat milk for 1 hour. The membrane was washed twice with PBST, with each wash taking 5 minutes. The membrane was subsequently incubated with PBST containing the horseradish peroxidase-conjugated secondary antibody and 1% nonfat milk for an hour. The washing was performed as described previously. The membrane was treated with the ECL Plus Western Blotting Detection Reagents (GE Lifesciences) as per manufacturer's instructions. The treated membrane was then exposed to X-ray film for visualization.

2.3 *S. cerevisiae*

2.3.1 Yeast strains and culture

Two yeast strains BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) and a YCA1 disruptant (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YOR197w::kanMX4) were kindly provided by Dr Norbert Lehming (University of Singapore, Singapore). These two strains were used as hosts for transformation.

Yeast cells were incubated at 30°C, and liquid cultures were shaken at 220 rpm. Fresh cultures of host strains were used for each set of transformation, by streaking out from stocks stored at -80°C, and then rendering the streaked yeast cells competent for transformation.

2.3.2 Yeast shuttle plasmid vectors

Three yeast shuttle vectors were used, PactTHA423, Pgal1-HA-PL-Tactin-423, and pESC-HIS (Stratagene). Both PactTHA423 and Pgal1-HA-PL-Tactin-423 were kindly provided by Dr Norbert Lehming (National University of Singapore, Singapore). PactTHA423 possesses the actin promotor-terminator cassette, resulting in constitutive protein expression. Pgal1-HA-PL-Tactin-423, on the other hand, possesses a Gal1 promotor, and protein expression is only induced in the presence of galactose. Both PactTHA423 and Pgal1-HA-PL-Tactin-423 will produce fusion proteins with a haemagluttin (HA) tag at the C-terminus. pESC-HIS contains an galactose-inducible promotor as well, and results in a fusion protein with a FLAG tag at the C-terminus. All three plasmids contain the ampicillin resistance gene for selection in bacteria and the HIS3 auxotrophic selection marker (yeast cells that have been successfully transformed with these plasmids are able to grow in histidine-deficient media).

For generation of HA-tagged fusion proteins using PactTHA423 and Pgal1-HA-PL-Tactin-423 plasmid vectors, PCR primers were designed to include an EcoRI restriction site at the 5'-end, and a SalI restriction site at the 3'-end of the PCR product. Similarly, generation of FLAG-tagged proteins using the pESC-HIS plasmid vector required PCR primers which incorporated an EcoRI restriction site at the 5'-end and a NotI restriction site at the 3'-end of the PCR product.

2.3.3 Isolation of yeast genomic DNA

Genomic DNA from wild-type *S. cerevisiae* strain BY4741 was obtained using the protocol of Harju *et. al* (2004). Briefly, a yeast colony was cultured overnight in 5 ml of YPDA medium. A 1.5 ml aliquot was centrifuged at maximum speed in a table-top micro-centrifuge for 5 minutes. The cell pellet was resuspended in 200 µl of Harju buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA). Cells were lysed

by immersing the tubes in liquid nitrogen for 2 minutes, and then transferred to a 95°C water bath for 1 minute. The freeze-thawing was repeated another two more times, following which the solution was vortexed for 30 seconds. 200 µl of chloroform was added, and mixed by gentle inversion. The mixture was centrifuged at maximum speed in a table-top micro-centrifuge for 3 minutes. The upper aqueous phase was transferred to a fresh micro-centrifuge tube, and 400 µl of ice-cold absolute ethanol was added. After mixing by gentle inversion, the mixture was incubated at -20°C for an hour. The precipitated DNA was recovered by centrifugation at maximum speed in a table-top micro-centrifuge for 5 minutes. The DNA pellet was washed with 70% ethanol, and air-dried. Once dry, the DNA pellet was resuspended in 50 µl of sterile deionised water.

2.3.4 PCR amplification of metacaspase gene YCA1

The following primers were used to amplify the YCA1 gene from *S. cerevisiae* genomic DNA: 5'YCA1-EcoRI (GCCGAATTCATGTATCCAGGTAGTGGAC) and 3'YCA1-SalI (GGGCGTCGACTACATAATAAATTGCAGATTTA), with the EcoRI and SalI restriction sites underlined respectively. Subsequently, the reverse primer was modified to include a hexahistidine tag at the C-terminus of the YCA1 protein: 3'-YCA1-6×His-SalI (GCGTCGACTA**GTGATGATGGTGATGATG**CATAATAAATTGCAGATTTACG). The SalI restriction site is underlined, while the nucleotide sequence for the hexahistidine tag are in bold.

PCR was performed using the Expand High Fidelity PCR kit (Roche) using the following conditions: initial denaturation was carried out at 95°C for 1 minute; 5 cycles of denaturation at 95°C for 1 minute, annealing at 51°C for 1 minute, and elongation at 72°C for 2 minutes; 25 cycles of denaturation at 95°C for 1 minute, annealing at 51°C for 1 minute, and elongation at 72°C for 2 minutes, with the duration for the elongation step increased by 5 seconds every cycle; elongation at 72°C for 7 minutes; a final hold step at 16°C.

2.3.5 Molecular cloning

Molecular cloning was carried out as described in section 2.1.10.

2.3.6 DNA sequencing

DNA sequencing was carried out as described previously for the PfMCA1 gene (section 2.1.11).

Plasmid Vector	Name	DNA Sequence
PactTHA423	YCA1-Pact-5'-2296	CTCACCCTAACATATTTTCCAATTAAC
	YCA1-Pact-5'-2700	CTTACTGCTTTTTTCTTCCCAAG
	YCA1-Pact-5'-3100*	GGTCCACCCCAGAATATGTCATTACCTC
	YCA1-Pact-5'-3500**	TTATATATCCGGTCGATTTCGAAACTC
	YCA1-Pact-3'-3350‡	ACCAAATCGTTCTGATCATCAG
	YCA1-Pact-3'-3750‡‡	AGCAGCCCTGTTTCCTGTGGCATATG
	YCA1-Pact-3'-4150	GTTTAAAAATAGAAATAGAGAGAGAGGTAC
	YCA1-Pact-3'-4486	GTATCAAAACGCCGGACTCA
Pgal1-HA-PL-Tactin-423	Pgal-5'	AAATCCACATAACTGACAAAACTGG
	YCA1-Pgal-5'-3740	GCTGTCGAAGATGGGCAAAATAC
pESC-HIS	YCA1-pESC-fw-4243	CAACATATAAGTAAGATTAGATATGGATATG
	YCA1-pESC-fw-4704*	GGTCCACCCCAGAATATGTCATTACCTC
	YCA1-pESC-fw-5104**	TTATATATCCGGTCGATTTCGAAACTC
	YCA1-pESC-rv-4933‡	ACCAAATCGTTCTGATCATCAG
	YCA1-pESC-rv-5329‡‡	AGCAGCCCTGTTTCCTGTGGCATATG
	YCA1-pESC-rv-5727	GATAAGATCTGAGCTCTTAATTAACAATTC

Table 3. List of sequencing primers used for the various clones of YCA1. As the sequence used is the same for all three plasmid vectors, only the first and last sequencing primer was changed. In the case of pESC-HIS, the change in name is merely cosmetic. Primers with the same sequence have the same symbol after their names. The number at the end represents the position of the primer.

2.3.7 Isolation of yeast total RNA

Total RNA was isolated from yeast strains according to the protocol of Li *et. al* (2009). Briefly, yeast strains were grown in 3 ml of the appropriate media, and approximately 2.5 OD_{600} of yeast culture were harvested by centrifugation. The cell pellet was washed in 400 µl of DEPC-treated water, before centrifugation at 12,000 rpm for 2 minutes. The cell pellet was resuspended in 400 µl of RNA isolation buffer (10 mM EDTA, 50 mM Tris-HCl, 5% SDS, pH 6.0). The suspension was incubated in a waterbath at 65^{o}C for 5 minutes, following which it was cooled rapidly in ice/water. 200 µl of 0.3 M KCl (pH 6.0) was added to the treated cell suspension, and mixed thoroughly to precipitate the SDS. The mixture was centrifuged at 12,000 rpm, 4^{o}C for 10 minutes. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the supernatant, and mixed by inversion, before centrifugation at 12,000 rpm, 4^{o}C for 5 minutes. The aqueous layer was recovered and precipitation was achieved by addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5

volumes of absolute ethanol. The mixture was then incubated at -20°C for 10 minutes. The precipitated RNA was pelleted by centrifugation at 13,000 rpm for 10 minutes at 4°C. The pellet was washed with 70% ethanol, and centrifuged at 13,000 rpm for 5 minutes at 4°C. The pellet was then air-dried before being resuspended in 50 µl of DEPC-treated water.

2.3.8 Quantification of yeast total RNA

Yeast total RNA was quantified as described previously for *P. falciparum* total RNA (section 2.1.4).

2.3.9 Preparation of yeast cDNA

Yeast total RNA was treated with DnaseI (Promega) according to manufacturer's instructions. 100 ng of the Dnase-treated total RNA was then used for first-strand cDNA synthesis using the RevertAid™ H-minus M-MuLV reverse transcriptase from Fermentas (according to the manufacturer's protocol), and oligo-dT primers. The reaction mixture was incubated for 60 minutes at 42°C. 4µl of the mixture was then used for PCR.

2.3.10 Preparation of competent yeast cells

Transformation was performed according to manufacturer's (Stratagene) instructions. Briefly, an overnight yeast culture was diluted 20× in YPDA medium to a total volume of 50 ml. The diluted culture was incubated for 4-5 hours before centrifugation at 1,000g for 5 minutes. The cell pellet was resuspended in 10 ml of LTE buffer (0.1 M LiOAc, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA) and centrifuged again at 1,000g for 5 minutes. The cell pellet was resuspended in 0.5 ml of LTE buffer, and kept at 4°C for up to 3 days.

2.3.11 Transformation

3 µl of recombinant plasmid solution (prepared using QIAprep Spin Miniprep Kit) and 60 µl of Transformation Mix (40% polyethylene glycol 3350, 0.1 M LiOAc, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA) was added to 10 µl of the competent yeast cell suspension. The mixture was gently inverted several times for homogenisation. The mixture was then incubated at 30°C for 30 minutes, after which it was heated at 42°C for 15 minutes. The mixture was then centrifuged at 1,000g for 3 minutes, and the pellet resuspended in 100 µl of distilled water, before being plated onto histidine-deficient agar plates, and incubated at 30°C.

2.3.12 Induction of protein expression

A yeast colony was picked and inoculated in 2 ml of non-inducing selective media (containing glucose). The culture was grown overnight in a shaking incubator at 220 rpm and 30°C. An aliquot of the overnight culture was added to 5 ml of fresh non-inducing selective media to OD_{600}=0.05. The diluted culture was incubated in a shaking incubater at 220 rpm, 30°C to an OD_{600} of 0.4-0.6. The culture was then centrifuged at 2,000 rpm for 10 minutes, and the cell pellet resuspended in an equal volume of the appropriate media (non-inducing, containing glucose as a carbon source or inducing, containing galactose). The resuspended cultures were then incubated overnight.

2.3.13 Preparation of yeast protein extracts

Yeast protein extracts were prepared according to the protocol of Kushnirov (2000). Briefly, approximately 2.5 OD_{600} of yeast cells were harvested from overnight cultures. The yeast cells were pelleted by centrifugation at 2,000 rpm for 10 minutes, and the cell pellet was resuspended in 100 µl of distilled water, before being transferred to a 1.5 ml tube. 100 µl of 0.2 M NaOH was added, and the suspension was incubated at room temperature for 5 minutes. The suspension was centrifuged in a table-top microcentrifuge at 2,000g for 2 minutes. The cell pellet was resuspended in 50 µl of SDS sample buffer (0.06 M Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 4% β-mercaptoethanol, 0.0025% bromophenol blue), and boiled at 100°C for 3 minutes. The boiled suspension was centrifuged at 13,000 rpm for 3 minutes, and 6 µl of the supernatant was used for SDS-PAGE.

2.3.14 Purification of hexahistidine-tagged proteins

1 ml Histrap HP columns (GE Healthcare) were used to purify hexahistidine-tagged proteins via immobilized metal ion adsorption chromatography (IMAC), as per manufacturer's instructions. Briefly, proteins extracts were prepared as described above in section 2.3.13, except the sample buffer did not contain any bromophenol blue. The sample was diluted 100× in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 30 mM imidazole, pH 7.4), and the diluted sample filtered using a 0.22 µm syringe filter. The column was washed with 5 column volumes of sterile distilled water, and equlibrated with 5 column

volumes of binding buffer. The filtered sample was applied to the column, after which 15 column volumes of binding buffer was used for washing. 5 column volumes of elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4), was used to elute the bound protein.

The eluted protein was then concentrated using the Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-3 membrane (Millipore), following manufacturer's instructions. Briefly, the eluted fraction was loaded onto the centrifugal unit in 2 ml aliquots, and spun at 3,500g for 10 minutes. Once the eluted protein has been concentrated, 100 µl of SDS sample buffer was used to recover the retentate, and used for immunoblotting.

2.3.15 Immunoblotting

Immunoblotting was performed as described in section 2.2.8.

2.3.16 Cell viability assays

Transformed yeast strains were grown overnight in the appropriate medium, and an aliquot of the overnight culture was added to fresh medium to an OD_{600} of 0.05. The freshly diluted medium was incubated at 30°C, 220 rpm in a shaking incubator until the OD_{600} had reached 0.4 to 0.6. An aliquot of the cell culture was then transferred to test media used in the cell viability assays such that the final cell density was 3×10^5 cells/ml. Typically, 30 µl is added for every 1 ml of the test medium.

2.3.16.1 Acetic acid assay

The acetic acid assay was performed as described by Ludovico *et al.* with modifications (Ludovico *et al*., 2001). Briefly, acetic acid was added to the appropriate yeast medium to a final concentration of 80 mM, and pH was adjusted to 3.0 with HCl. Yeast cells were introduced into media containing acetic acid at the first timepoint 0 minute. In total, there were 5 timepoints – 0, 30, 60, 120, and 200 minutes. At each timepoint, a 1 µl aliquot was taken and diluted 1,000×. 100 µl of the diluted sample was plated onto histidine-deficient agar plates. The plates were then incubated in a 30°C incubator for 2 days, before the number of colony forming units (CFU) was determined by visual counting.

2.3.16.2 Hydrogen peroxide assay

A modified protocol of Madeo *et al.*, (1999) was used for this assay. Briefly, H_2O_2 was added to the appropriate yeast medium to a final concentration of 3 mM. Yeast cells were introduced into media containing H_2O_2 at the first timepoint 0 minute. The timepoints used for this assay are the same as that described for the acetic acid assay. Similarly as well, at each timepoint, a 1 µl aliquot was taken and diluted 1,000×. 100 µl of the diluted sample was plated onto histidine-deficient agar plates. The inoculated plates were then incubated in a 30°C incubator for 2 days, before the number of colony forming units (CFU) was determined by visual counting.

2.3.16.3 Hyperosmotic shock assay

Yeast cells were introduced into the appropriate yeast medium containing 60% (w/w) glucose at the first timepoint 0 hour. At each timepoints of 0, 1, 2, 4, 6, 8 and 10 hours, 1µl aliquots were taken and diluted before being plated onto agar plates, as described previously for the acetic acid and H_2O_2 assays. The inoculated agar plates were incubated at 30°C for 2 days before determination of CFU.

2.4 *Trypanosoma brucei*

2.4.1 Trypanosome strains and culture

Procyclic *T. brucei brucei* strain 29.13 cells were used for RNA interference studies and stable transfection. They were grown in Cunningham medium with 15% heat-inactivated fetal calf serum in the presence of 30 µg/ml phleomycin at 28°C. Procyclic *T. brucei rhodesiense* YTAT cells were used for transient recombinant protein expression. These cells were grown in Cunningham medium with 15% heat-inactivated fetal calf serum in the presence of 10 µg/ml of blasticidin at 28°C. Fresh media was changed every other day.

2.4.2 Plasmids

The p2T7, pLEW100 and pXS2 plasmids were kindly provided by Dr Cynthia He (National University of Singapore, Singapore). The p2T7 plasmid was used for RNAi studies

of the trypanosome metacaspase 4 (TbMCA4). DNA sequences were cloned into the p2T7 plasmid by bidirectional cloning utilising XbaI restriction sites.

The pXS2 plasmid utilizes the yellow fluorescent protein (YFP) reporter system – YFP will be attached at the C-terminus of the cloned gene. Cloning was achieved by having the NheI restriction site and BamHI restriction site at the 5'-end and 3'-end of the sequence respectively.

Transgene expression using the pLEW100 vector is regulated by a tetracycline-inducible system, and stable integration of the cloned gene was achieved by electroporating competent *T. brucei* cells with linearized fusion pLEW100 plasmids. HindIII and BamHI restriction sites were used for cloning of PfMCA1 into the plasmid vector.

2.4.3 Isolation of *T. brucei* genomic DNA

Cells were harvested from a 10 ml culture of *T. brucei* strain YTAT cells by centrifugation at 3,500 rpm for 10 minutes at 4°C. The supernatant was discarded and the cell pellet was washed with 1 ml of TE buffer. The cell pellet was resuspended in 0.5 ml of TE buffer, and 20 µl of 10% SDS, 10 µl of proteinase K (from a stock of 10 µg/µl solution), and 10 units of RNase was added. The mixture was homogenised and incubated at 55°C for an hour. DNA was extracted and precipitated from the aqueous layer, as described in section 2.1.2. The DNA pellet was resuspended in 50 µl of sterile deionised water.

2.4.4 Electroporation

1×10^7 *T. brucei* cells were pelleted at 3000g for 7 minutes and washed in 0.5 volumes of cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4,25 mM HEPES, 2 mM EGTA, 5 mM $MgCl_2$, pH 7.6). The suspension was centrifuged again, and the pellet was resuspended in 1 ml of cytomix. 15 µg of linearized plasmid DNA (stable transfection) or 50 µg of circular plasmid DNA (transient transfection) was added to 0.5 ml of the cell suspension in a 0.4 cm electroporation cuvette. Electroporation was carried out twice at 1500 V and 25 µF, with a 10-second pause between the two electroporations. The mixture was then added to 10 ml of Cunningham's media containing 10 µg of phleomycin, and incubated at 27°C.

2.4.5 Molecular cloning

Molecular cloning was carried out as described in section 2.1.10.

2.4.6 RNA interference of TbMCA4

The sequence of TbMCA4 (systematic name Tb10.70.5250) was obtained via GeneDB (http://www.genedb.org/), and the fragment to be cloned into the RNAi vector was designed by entering the gene sequence into the TrypanoFAN website (http://trypanofan.path.cam.ac.uk/software/RNAit.html). The query returned primer sequences which amplified a portion of the gene from positions 517 to 1023. These primer sequences were modified to include the XbaI restriction sequence for cloning into the p2T7 plasmid vector.

The recombinant p2T7 plasmid was linearized using NotI, and the linearized plasmied was introduced into *T. brucei* strain 29.13 via electroporation. After electroporation, the cells were kept in Cunningham medium containing 15% heat-inactivated fetal calf serum in the presence of 30 µg/ml phleomycin at 28^{o}C. The presence of tetracycline at a concentration of 10 µg/ml causes RNA interference of the targeted gene.

2.4.7 Clonal selection

T. brucei cells that were successfully transfected were used for clonal selection. 2×10^{6} cells were diluted 100× in fresh media twice, and 200 µl of the diluted sample was added to 20 ml of fresh media containing 20 µl of phleomycin and 5000 *T. brucei* strain YTAT cells. 200 µl of the mixture was added to each well of a 96-well plate, and the plate sealed with paraffin tape. The plate was incubated at 27^{o}C for 2 weeks. Positive clones were identified by growth and a change in media colour from red to yellow.

Four of the positive clones were selected and grown in 10 ml of fresh Cunningham media with the presence of phleomycin. These clones were grown for 2-3 days to ensure that they were viable. From each clone, 2×10^{7} cells were harvested, and inoculated into 10 ml of fresh Cunningham media with phleomycin. A 10 µl aliquot of the culture was taken every 24

hours, for 4 days, and diluted 10× in PBS buffer. 10 µl of the diluted sample was used for cell counting using a Neubauer haemocytometer.

2.4.8 Isolation of *T. brucei* total RNA for reverse-transcriptase PCR

A freshly inoculated culture was incubated overnight, and 2×10^7 cells from the overnight culture were resuspended in 1 ml of fresh medium. Resuspended cultures were transferred to a 24-well plate, and tetracycline was added to the culture medium for induction of RNAi. 50% ethanol was used as a vehicular control. The plate was incubated at 28°C for 2 hours, before cells were harvested for total RNA with Trizol (Invitrogen), as per manufacturer's instructions.

RNA quantification and RT-PCR was carried as described in sections 2.3.8 and 2.3.9 respectively.

2.4.9 Concanavalin A treatment

A freshly inoculated 10 ml culture was incubated overnight, and the cell count adjusted to 2×10^7 cells per ml. 2 ml of the adjusted culture was aliquoted into each well of a 6-well plate. Concanavalin A (ConA) was added to the desired concentration, and a 10 µl aliquot was used for cell counting using a Neubauer haemocytometer every 24 hours, for 96 hours.

3. RESULTS

3.1 Homology of PfMCA1

YCA1 has been shown to be involved in the attenuation of cell death in *S. cerevisiae* induced by hydrogen peroxide and acetic acid (Ludovico *et al*., 2001) etc. The protein sequence of YCA1 was used in a BLAST search against a *P. falciparum* 3D7 protein database, identifying a putative caspase protein homologue, PF13_0289. PF13_0289 (PfMCA1) consists of 1,842 bp, and encodes 613 amino acids with a predicted molecular weight of 71.7 kDa. A BLAST search using the full-length PfMCA1 protein sequence reveals that it has a 42% identity with YCA1. PfMCA1 possesses a universally conserved caspase domain from amino acid positions 318 to 551, and the critical histidine and cysteine residues which make up the histidine-cysteine dyad of caspases are at positions 404 and 460 of PfMCA1 respectively (Fig. 4).

PfMCA1 is predicted to cleave into two fragments upon processing (Meslin *et al*., 2007). Similarly, YCA1 has been shown to be processed in a manner not unlike metazoan caspases (Madeo *et al*., 2002). It is unknown whether this cleavage event is autocatalytic, or it has to be initiated by an unknown protease. Analysis of the N-terminal prodomain revealed a CARD domain, suggesting that upstream signals regulate the activation of the protein.

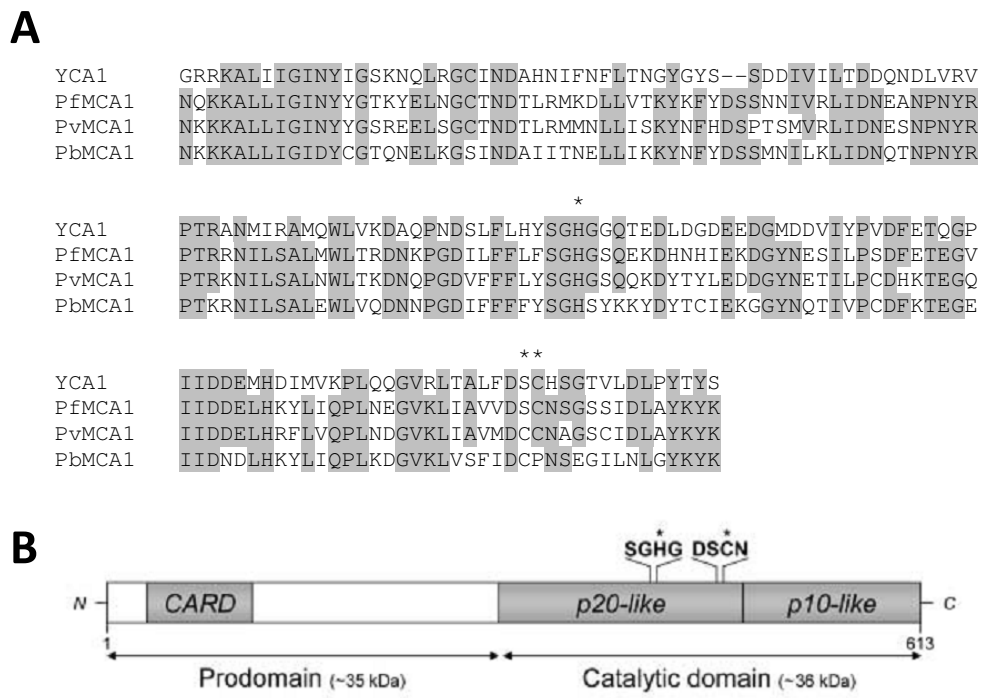

Figure 4. *In silico* studies of PfMCA1.

A. ClustalW mutilple alignment (using default parameters) of conserved portions of Peptidase_C14/Caspase domains of YCA1, PfMCA1, *Plasmodium vivax* MCA1 (PvMCA1) and *Plasmodium berghei* MCA1 (PbMCA1). Amino acid identity shared by 3 or more of the aligned sequences are shaded. Asterisks denote the critical active site histidine and cysteine residues of the catalytic dyad. Sequences are obtained from PlasmoDB (http://plasmodb.org/plasmo/home.jsp): PV114725 (PvMCA1), PB001074 (PbMCA1). Adapted from Le Chat L *et al*., 2007.

B. Predicted struture of PfMCA1. The predicted prodomain contains a putative CARD domain, and the active site histidine and cysteine residues are denoted by asterisks. Adapted from Meslin *et al.* 2007.

3.2 Expression of PfMCA1 and YCA1 protein in yeast

Once the two metacaspases genes had been successfully cloned into the respective plasmid vectors, and had been verified by DNA sequencing, the recombinant plasmids were used to transform the following yeast host cells, wild-type (WT) and YCA1-knockout (ΔYCA). Initial attempts to clone the metacaspase genes used PactTHA423 as the plasmid vector, and detection of fusion proteins was via the HA tag.

Attempts to detect the HA-tagged metacaspase proteins via immunoblots did not shown any significant bands at the expected positions. The expected size of PfMCA1 is

approximately 72 kDa and that of YCA1 is approximately 48 kDa. Repeats of the experiments using fresh cells showed the same results.

To eliminate the possibility that the failure to detect the fusion proteins were due to the epitope tags, and that the antibodies were not successfully binding to the HA-tag, a hexahistidine tag was engineered into the metacaspase gene sequences. This would result in six consecutive histidine residues after the metacaspase coding sequence, and this could be detected by anti-hexahistidine antibodies. Furthermore, overexpression of a protein can often result in the protein being stored in inclusion bodies; fusion tags such as the hexahistidine tag, have been shown to enhance the solubility of the fusion protein (Birkholtz *et al*., 2008; Esposito and Chatterjee, 2006), and hopefully allow the detection of the tagged protein. As the hexahistidine tag will bind readily to metal ions, any fusion protein can be purified via immobilized metal ion adsorption chromatography (IMAC). However, no tagged proteins could be detected from the immunoblots, nor from IMAC-purified fractions, suggesting that the proteins themselves were not well-tolerated by the yeast host cells.

The possibility exists that the expressed metacaspase proteins were toxic to the yeast host cells, and were therefore rapidly degraded. The metacaspase proteins were thus expressed using an inducible promotor. The metacaspase cloning sequences were cloned into the Pgal1-HA-PL-Tactin-423 plasmid vector, and protein expression was induced in the presence of galactose. Yeast cells were grown to a predetermined optical density, and the carbon source in the medium was changed for protein induction.

Despite the change from a constitutive to an inducible promotor, no fusion proteins could be detected from immunoblots, either using the HA or the hexahistidine tags. Surprisingly, no YCA1 fusion proteins could be detected, even though it is a protein endogenous to yeast, and previous studies have shown that it is possible to overexpress YCA1 in yeast cells (Bettiga *et al*., 2004; Madeo *et al*., 2002; Watanabe and Lam, 2005).

3.3 Optimization of protein expression

80.6% of the *P. falciparum* genome consists of adenosines and thymidines, making it one of the most (A+T)-rich genomes ever sequenced to date. The coding sequences of *P. falciparum* genes have an average length of 2.3 kilo base-pairs, larger than other organisms in which the gene lengths range from 1.3 to 1.6 kilo base-pairs (Gardner *et al*., 2002). When expressed in *E. coli* or yeast, the high (A+T)-content of *P. falciparum* genes could result in fortuitous polyadenation or transcription termination signals, leading to undesired truncated mRNA transcripts and low levels of mRNA. In addition, the high (A+T)-content translates to a codon usage that is dominated by adenosines and thymidines, making it extremely biased (Withers-Martinez *et al*., 1999; Yadava and Ockenhouse, 2003; Zhang *et al*., 2002). These factors combine to make heterologous expression in prokaryotic and eukaryotic systems an exceptionally difficult task. However, this situation is not unique to *P. falciparum*; other organisms such as *Clostridium tetani* (Romanis *et al*., 1991) and *Corynebacterium diphtheriae* (Woo *et al*., 2002) also face the same problems.

In order to circumvent the obstacles that prevent PfMCA1 expression in *S. cerevisiae*, the coding sequence of PfMCA1 was codon-optimized for yeast expression, and any long nucleotide sequences containing adenosines and thymidines which might be recognized as termination sequences were kept to a minimum. In addition, any glycosylation motifs recognized by *S. cerevisiae* were removed, and the (A+T)-content was increased as much as possible to aid protein expression. The optimized coding sequence decreased the (A+T)-content from 76.35% to 67.32%. While a 9% change may not seem significant by any measure, together with the incorporation of a yeast codon bias, it may be sufficient for protein expression to be observed.

```
  1  ATG                         GAA AAA ATA TAC GTC AAA ATA TAT GAA  30
  1  ATG CAC CAC CAT CAC CAT CAT GAG AAA ATT TAT GTC AAG ATT TAC GAA  48
  1  Met His His His His His His Glu Lys Ile Tyr Val Lys Ile Tyr Glu  16

 31  TTG TCT GGA TTA GAA GAT AAG GAT AAT TTT TCA TGT TAT ATA AAA ATA  78
 49  TTG AGT GGA CTG GAA GAT AAA GAT AAC TTC AGT TGT TAT ATC AAA ATC  96
 17  Leu Ser Gly Leu Glu Asp Lys Asp Asn Phe Ser Cys Tyr Ile Lys Ile  32

 79  TAT TGG CAG AAT AAG AAA TAT AAA AGT TGT ATA CTT CAA AAG AAT CCA  126
 97  TAC TGG CAA AAT AAG AAA TAT AAG TCA TGT ATC TTG CAG AAG AAT CCA  144
 33  Tyr Trp Gln Asn Lys Lys Tyr Lys Ser Cys Ile Leu Gln Lys Asn Pro  48

127  TAT AAA TTT AAT GAA ATC TTT TTA TTA CCT ATA GAC ATA AAA AAT AAT  174
145  TAC AAG TTT AAC GAA ATC TTG TTG CTC CCT ATC GAT ATT AAA AAT AAT  192
 49  Tyr Lys Phe Asn Glu Ile Phe Leu Leu Pro Ile Asp Ile Lys Asn Asn  64

175  GTT AAA GAT GAG AAA AAT AAT ATT TTG TCC ATT GAA GTA TGG TCC AGT  222
193  GTT AAA GAT GAG AAG AAT AAT ATC CTT TCT ATC GAG GTT TGG TCT TCC  240
 65  Val Lys Asp Glu Lys Asn Asn Ile Leu Ser Ile Glu Val Trp Ser Ser  80

223  GGT ATA TTA AAT AAT AAT AAA ATA GCC TAT ACC TTT TTT GAG CTC GAT  270
241  GGT ATC TTG AAT AAT AAT AAG ATT GCA TAT ACT TTC TTT GAG TTA GAT  288
 81  Gly Ile Leu Asn Asn Asn Lys Ile Ala Tyr Thr Phe Phe Glu Leu Asp  96

271  CAT ATT AGA AGA GAA AGA ATA TCA AGT GAA AAG ATT AAT TTG ATT GAT  318
289  CAC ATC AGA AGG GAG CGT ATA TCA AGC GAA AAG ATT AAC CTT ATA GAT  336
 97  His Ile Arg Arg Glu Arg Ile Ser Ser Glu Lys Ile Asn Leu Ile Asp  112

319  GTT GTA AAG AAA TGT ACA TTG CAA ATA TCT GTT CAT ATA ATA AAT AAT  366
337  GTC GTC AAG AAA TGT ACA CTA CAA ATT AGT GTC CAT ATT ATG AAT AAC  384
113  Val Val Lys Lys Cys Thr Leu Gln Ile Ser Val His Ile Ile Asn Asn  128

367  AAT CAA GAT ATC CTA TTT TGT AAT ATA AAA GAT ATA TTT GGT AAT AAT  414
385  AAC CAG GAT ATT CTG TTT TGC AAC ATC AAA GAC ATA TTC GGT AAC AAT  432
129  Asn Gln Asp Ile Leu Phe Cys Asn Ile Lys Asp Ile Phe Gly Asn Asn  144

415  AAA AAT GAT AAA GAA ATA CAT GAT GCC ATA TTA AAA TAT GGA GGT AAT  462
433  AAG AAC GAT AAA GAG ATT CAT GAC GCT ATT TTG AAA TAT GGA GGT AAC  480
145  Lys Asn Asp Lys Glu Ile His Asp Ala Ile Leu Lys Tyr Gly Gly Asn  160

463  GAA AGG CAT ATA ATT AAG GAA CTT CGT AAA GAA AAG GAA ATT GGA CAA  510
481  GAA AGG CAC ATT ATC AAG GAA TTA AGA AAA GAG AAG GAG ATT GGA CAG  528
161  Glu Arg His Ile Ile Lys Glu Leu Arg Lys Glu Lys Glu Ile Gly Gln  176

511  TAT AAT AAT ATA TAT TTT AAT GAT TAT GTA AAT GTT CTT AAT ACT GAT  558
529  TAT AAC AAT ATA TAC TTT AAC GAT TAT GTC AAC GTT CTG AAT ACT GAT  576
177  Tyr Asn Asn Ile Tyr Phe Asn Asp Tyr Val Asn Val Leu Asn Thr Asp  192

559  CCA TCT CAG AAT TAT ATA TAT AAT GAT ATG CCT AAA ATT ACA CCA AAT  606
577  CCT TCT CAG AAT TAT ATC TAC AAC GAT ATG CCG AAG ATT ACA CCG AAT  624
193  Pro Ser Gln Asn Tyr Ile Tyr Asn Asp Met Pro Lys Ile Thr Pro Asn  208
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Figure 5. Optimization of the PfMCA1 gene sequence for yeast expression. The original PfMCA1 gene sequence, the optimized PfMCA1 gene, and the PfMCA1 amino acid sequence are shown in black, green and blue respectively. In the optimized sequence, the nucleotides which have been changed are highlighted in black. In addition, a hexhistidine tag has been added after the start codon in the optimized gene sequence. The critical histidine and cysteine residues of the catalytic dyad are boxed in grey.

```
 607  AAT ATA TAT AAT AAT ATG AAT AAT GAT CAA ACA AAT CAT ACA TAT TTA  654
 625  AAT ATC TAT AAT AAC ATG AAT AAC GAT CAG ACT AAT CAT ACA TAT CTT  672
 209  Asn Ile Tyr Asn Asn Met Asn Asn Asp Gln Thr Asn His Thr Tyr Leu  224

 655  AAA GCA CCT AAT AGT TTA TAT AAT AAC GAA AAC ACA ATT TAT TCA TCT  702
 673  AAA GCA CCA AAT TCA CTA TAC AAT AAT GAA AAT ACT ATC TAC TCT AGT  720
 225  Lys Ala Pro Asn Ser Leu Tyr Asn Asn Glu Asn Thr Ile Tyr Ser Ser  240

 703  AAT GTA CAT TAT AGC ACT TAT ATG AAC AAT TCA CCT ACT TAT AAA AAT  750
 721  AAT GTC CAT TAT AGC ACA TAC ATG AAT AAT AGT CCA ACT TAT AAA AAC  768
 241  Asn Val His Tyr Ser Thr Tyr Met Asn Asn Ser Pro Thr Tyr Lys Asn  256

 751  TCA AAT AAT ATG AAT CAT GTA ACA AAT ATG TAT GCA TCC AAT GAT TTA  798
 769  AGC AAT AAT ATG AAC CAC GTC ACA AAC ATG TAC GCA TCT AAT GAT TTA  816
 257  Ser Asn Asn Met Asn His Val Thr Asn Met Tyr Ala Ser Asn Asp Leu  272

 799  CAC AAT TCA AAT CAT TTT AAA CCT CAT AGT AAT GCA TAT AGC ACT ATA  846
 817  CAT AAC TCC AAT CAT TTC AAA CCT CAC TCT AAC GCA TAT TCG ACT ATT  864
 273  His Asn Ser Asn His Phe Lys Pro His Ser Asn Ala Tyr Ser Thr Ile  288

 847  AAT TAT GAT AAT AAT AAT TAT ATA TAT CCT CAA AAT CAT ACA AAT ATA  894
 865  AAC TAC GAT AAC AAT AAT TAT ATA TAT CCT CAA AAT CAT ACC AAC ATT  912
 289  Asn Tyr Asp Asn Asn Asn Tyr Ile Tyr Pro Gln Asn His Thr Asn Ile  304

 895  TAT AAT AGA GCA TCT CCT GGT AGT GAT CAA ACT TTA TAT TTT TCT CCA  942
 913  TAC AAT AGG GCT AGT CCG GGA AGT GAT CAA ACT TTA TAC TTC AGT CCA  960
 305  Tyr Asn Arg Ala Ser Pro Gly Ser Asp Gln Thr Leu Tyr Phe Ser Pro  320

 943  TGT AAT CAA AAG AAA GCA TTG CTT ATT GGG ATA AAT TAT TAT GGA ACC  990
 961  TGT AAC CAA AAG AAG GCA TTA CTG ATC GGT ATC AAT TAT TAC GGC ACG 1008
 321  Cys Asn Gln Lys Lys Ala Leu Leu Ile Gly Ile Asn Tyr Tyr Gly Thr  336

 991  AAA TAT GAA TTG AAT GGT TGT ACA AAT GAT ACA CTG AGA ATG AAA GAT 1038
1009  AAA TAT GAA CTG AAC GGC TGT ACT AAC GAT ACA CTT CGT ATG AAA GAT 1056
 337  Lys Tyr Glu Leu Asn Gly Cys Thr Asn Asp Thr Leu Arg Met Lys Asp  352

1039  TTG CTA GTA ACA AAA TAT AAA TTT TAT GAT TCC TCA AAT AAT ATA GTT 1086
1057  TTA TTA GTT ACA AAG TAC AAG TTT TAC GAT TCT TCT AAC AAC ATT GTT 1104
 353  Leu Leu Val Thr Lys Tyr Lys Phe Tyr Asp Ser Ser Asn Asn Ile Val  368

1087  AGA TTG ATT GAT AAC GAA GCA AAT CCA AAT TAT AGA CCT ACA AGA AGA 1134
1105  AGA CTA ATT GAC AAT GAA GCA AAC CCG AAT TAT AGA CCG ACA AGA AGA 1152
 369  Arg Leu Ile Asp Asn Glu Ala Asn Pro Asn Tyr Arg Pro Thr Arg Arg  384

1135  AAT ATT TTA TCA GCA CTT ATG TGG TTA ACT AGG GAT AAT AAA CCA GGA 1182
1153  AAT ATC TTA AGT GCC TTA ATG TGG TTG ACT AGA GAT AAC AAA CCT GGC 1200
 385  Asn Ile Leu Ser Ala Leu Met Trp Leu Thr Arg Asp Asn Lys Pro Gly  400

1183  GAT ATT TTA TTT TTC CTT TTT TCA GGA CAT GGA TCA CAA GAA AAA GAT 1230
1201  GAC ATT CTG TTC TTC CTA TTT TCT GGT CAC GGC TCT CAG GAG AAA GAT 1248
 401  Asp Ile Leu Phe Phe Leu Phe Ser Gly His Gly Ser Gln Glu Lys Asp  416

1231  CAT AAT CAT ATA GAA AAG GAT GGT TAT AAT GAA TCT ATT CTA CCG TCT 1278
1249  CAT AAT CAC ATT GAA AAG GAC GGT TAT AAC GAA TCT ATA TTG CCA TCA 1296
 417  His Asn His Ile Glu Lys Asp Gly Tyr Asn Glu Ser Ile Leu Pro Ser  432

1279  GAT TTT GAA ACA GAA GGT GTA ATT ATT GAT GAT GAA TTA CAT AAA TAT 1326
1297  GAC TTT GAG ACC GAG GGA GTT ATA ATC GAC GAT GAA TTG CAT AAG TAC 1344
 433  Asp Phe Glu Thr Glu Gly Val Ile Ile Asp Asp Glu Leu His Lys Tyr  448
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1327  TTA ATT CAA CCC TTA AAT GAG GGA GTA AAA TTA ATA GCT GTT GTA GAT  1374
1345  CTA ATT CAA CCA CTA AAC GAG GGA GTC AAA TTC ATT GCT GTT GTA GAT  1392
 449  Leu Ile Gln Pro Leu Asn Glu Gly Val Lys Leu Ile Ala Val Val Asp  464

1375  AGT TGT AAT TCT GGA AGT AGT ATT GAT TTA GCT TAT AAA TAT AAA TTA  1422
1393  TCG TGT AAT AGC GGT TCT TCT ATA GAC TTG GCT TAT AAG TAC AAG TTA  1440
 465  Ser Cys Asn Ser Gly Ser Ser Ile Asp Leu Ala Tyr Lys Tyr Lys Leu  480

1423  AAA TCA AAA AAA TGG AAA GAA GAC AAA AAT CCA TTC CAT GTA ATT TGT  1470
1441  AAA TCC AAA AAG TGG AAG GAA GAT AAG AAC CCT TTT CAC GTG ATT TGT  1488
 481  Lys Ser Lys Lys Trp Lys Glu Asp Lys Asn Pro Phe His Val Ile Cys  496

1471  GAT GTT ACA CAA TTT AGT GGA TGT AAA GAT AAG GAA GTT TCT TAT GAA  1518
1489  GAT GTT ACG CAA TTC TCT GGT TGC AAA GAC AAA GAA GTC AGC TAC GAA  1536
 497  Asp Val Thr Gln Phe Ser Gly Cys Lys Asp Lys Glu Val Ser Tyr Glu  512

1519  GTT AAC ACA GGA CAG ATT GCA CCA GGT GGA TCA TTA GTT ACA GCT ATG  1566
1537  GTA AAT ACT GGA CAA ATT GCA CCA GGT GGA TCA TTA GTT ACT GCT ATG  1584
 513  Val Asn Thr Gly Gln Ile Ala Pro Gly Gly Ser Leu Val Thr Ala Met  528

1567  GTA CAA ATT TTG AAA AAT AAT ATG AAT ACA CCT TCT ATT ATA ACT TAT  1614
1585  GTT CAA ATC TTG AAG AAT AAT ATG AAC ACT CCT TCC ATT ATT ACG TAT  1632
 529  Val Gln Ile Leu Lys Asn Asn Met Asn Thr Pro Ser Ile Ile Thr Tyr  544

1615  GAA TAC TTA TTA CAT AAT ATA CAT GCT CAT GTC AAA CAA CAT AGT AAT  1662
1633  GAA TAT TTG CTA CAT AAT ATC CAT GCT CAT GTA AAG CAA CAT AGC AAT  1680
 545  Glu Tyr Leu Leu His Asn Ile His Ala His Val Lys Gln His Ser Asn  560

1663  CAA ACT GTT ACT TTT ATG TCA TCT CAA AAA TTT AAC ATG AAT AGA CTA  1710
1681  CAG ACT GTT ACT TTT ATG TCT TCA CAA AAG TTC AAC ATG AAT AGA TTG  1728
 561  Gln Thr Val Thr Phe Met Ser Ser Gln Lys Phe Asn Met Asn Arg Leu  576

1711  TTC GAT TTT GAA CAT ATA ATT AAG AAC AAA AAT AAC CAA CTA GGG CAA  1758
1729  TTC GAT TTT GAA CAT ATT ATC AAG AAC AAG AAT AAC CAA CTT GGT CAA  1776
 577  Phe Asp Phe Glu His Ile Ile Lys Asn Lys Asn Asn Gln Leu Gly Gln  592

1759  ATA ATT AAT AAA TAT ATA GAA AAA AAT AAA AGC AAA AAT AAA AAT AAG  1806
1777  ATA ATT AAT AAA TAT ATC GAA AAG AAT AAA TCC AAG AAC AAA AAC AAG  1824
 593  Ile Ile Asn Lys Tyr Ile Glu Lys Asn Lys Ser Lys Asn Lys Asn Lys  608

1807  TTA AAG CAT GAA CTT AAA AAT TTA TTT TTT TTT                      1839
1825  CTT AAG CAT GAA TTC AAC AAT TTA TTT TTC TTC                      1857
 609  Leu Lys His Glu Leu Lys Asn Leu Phe Phe Phe                      619
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3.4 Expression of optimized PfMCA1 and YCA1 amplified from mRNA

The optimized PfMCA1 coding sequence was synthesized by Genscript, and was cloned into a commercial vector pESC-HIS (Stratagene). pESC-HIS was chosen as the plasmid vector, as it was identical to the one that was used in the initial study that overexpressed and characterized YCA1 (Madeo *et al*., 2002). Proteins expressed using pESC-HIS would express a FLAG epitope tag at the C-terminus.

However, the presence of any PfMCA1 could not be detected, using either anti-histidine or anti-FLAG antibodies (Fig. 6A). This suggested that the failure to express PfMCA1 is inherent to the protein itself, and was not due to the expression system.

To ensure that the yeast expression system was working, the coding sequence of *S. cerevisiae* actin was amplified via PCR from cDNA, using the primers EcoRI-ScActin-fw (GCCGAATTCATGGATTCTGAGGTT) and NotI-ScActin-rv (TATAGCGGCCGCGAAA-CACTTGTGGTG). The EcoRI and NotI restriction sites are underlined respectively. The *S. cerevisiae* actin coding sequence was cloned into pESC-HIS, and protein expression was induced. Immunoblotting using yeast transformed with the recombinant plasmid grown under inducing conditions (presence of galactose) showed the expected band at 41.7 kDa(Fig. 6B).

Since there did not seem to be any problems with the expression system, the failure to overexpress YCA1, an endogenous yeast protein, seems puzzling. In an attempt to address this anomaly, the coding sequence of YCA1 was amplified from *S. cerevisiae* cDNA. This was subsequently cloned into pESC-HIS, and immunoblots showed that the YCA1 gene had been successfully cloned and expressed (Fig. 7). This result suggested that a mRNA source might be better than a genomic DNA source for gene amplification and subsequent expression, despite the fact that both sequences are virtually identical, for all intents and purposes.

When YCA1 was overexpressed in yeast cells, a band of approximately 50 kDa, which likely correspond to the predicted size of the full-length protein, was detected. In addition, prominent bands averaging 37 kDa in size were also observed for transformed WT yeast strains (Fig. 7, lanes 3 & 7), but not in transformed ΔYCA1 yeast strains (Fig. 7, lanes 5

& 9). This second group of bands most likely corresponds to the catalytic domain of YCA1 (Madeo *et al*., 2002; Watanabe and Lam, 2005).

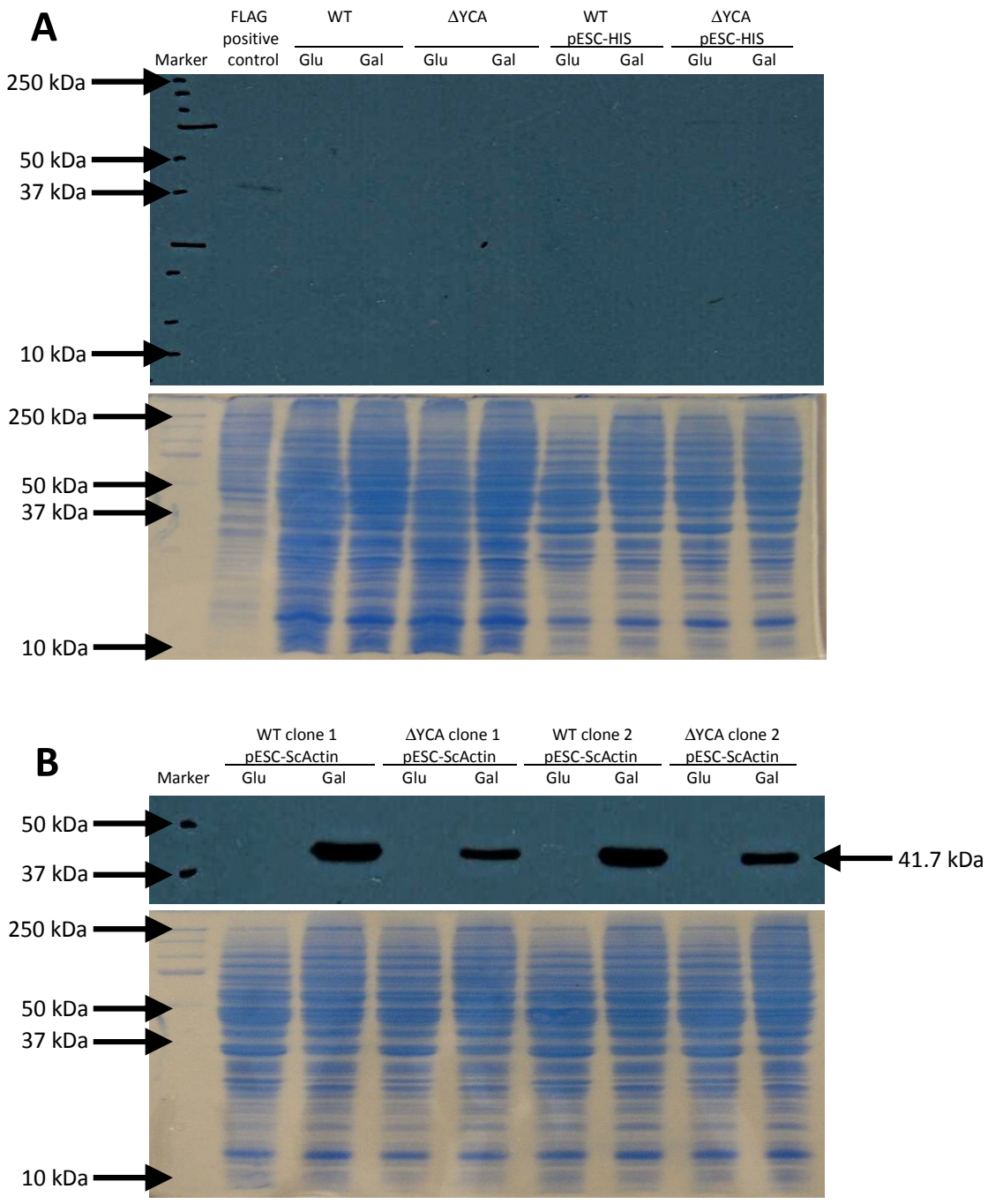

Figure 6. Overexpression of *S. cerevisiae* actin.
Cells were grown in non-inducing media containing glucose (Glu), and in inducing media containing galactose (Gal).
A. Positive and negative controls for immunoblot using anti-FLAG antibodies. FLAG-tagged CD74 (approximately 37 kDa) was used as the positive control for the antibody. Proteins were harvested from both WT and ΔYCA1 *S.crevisiae*, as well as strains transfomed with the empty pESC-HIS vector. Protein loading was determined by Coomassie Blue staining (shown below the immunoblot). Precision Plus Protein Dual Color standard (Bio-Rad) was used as the protein marker.
B. Detection of FLAG-tagged *S. cerevisiae* actin. The coding sequence for actin was cloned into pESC-HIS, and the recombinant plasmid (pESC-ScActin) was used to transform WT and ΔYCA1 *S. cerevisiae*. The FLAG-tagged actin was predicted to have a size of 41.7 kDa. Protein extracts were harvested and separated on an SDS-PAGE gel. Protein loading was determined by Coomassie Blue staining (shown below the immunoblot). Two clones were used for protein detection.

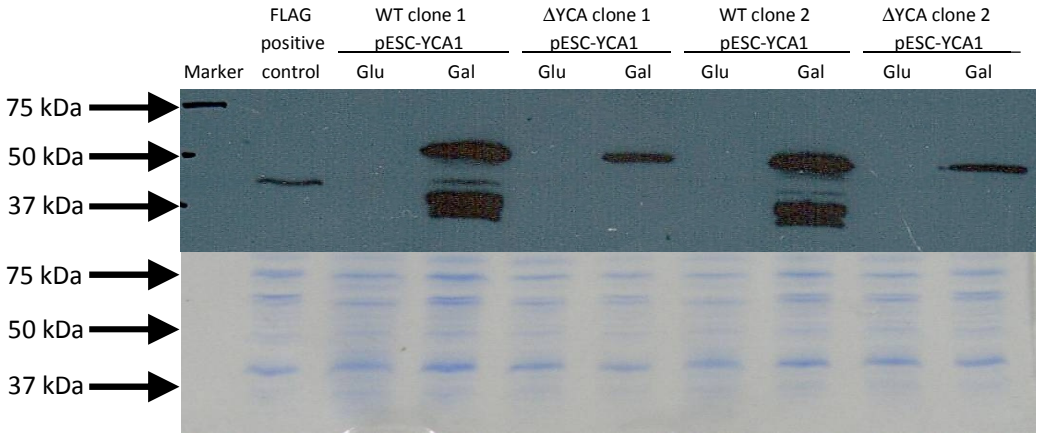

Figure 7. Overexpression of YCA1. Both WT and ΔYCA yeast were transformed with pESC-HIS containing the YCA1 coding sequence. Transformed yeast were grown in both non-inducing (Glu) and inducing media (Gal). Proteins were harvested and separated on a SDS-PAGE gel. Two clones each were used for protein detection.

3.5 PfMCA1 mRNA levels in transformed yeast

To further understand why PfMCA1 could not be expressed in *S. cerevisiae*, RNA was isolated from both WT and ΔYCA1 yeast transformed with the full-length PfMCA coding sequence. Using oligo-dT primers, the mRNA fraction of the total RNA pool was converted into cDNA. The cDNA was used for PCR amplification using *S. cerevisiae* actin-specific and PfMCA1-specific primers (Fig. 8). Actin-specific primers were used as a control for the RT-PCR reaction, while PfMCA1-specific primers were used to detect any PfMCA1 transcripts.

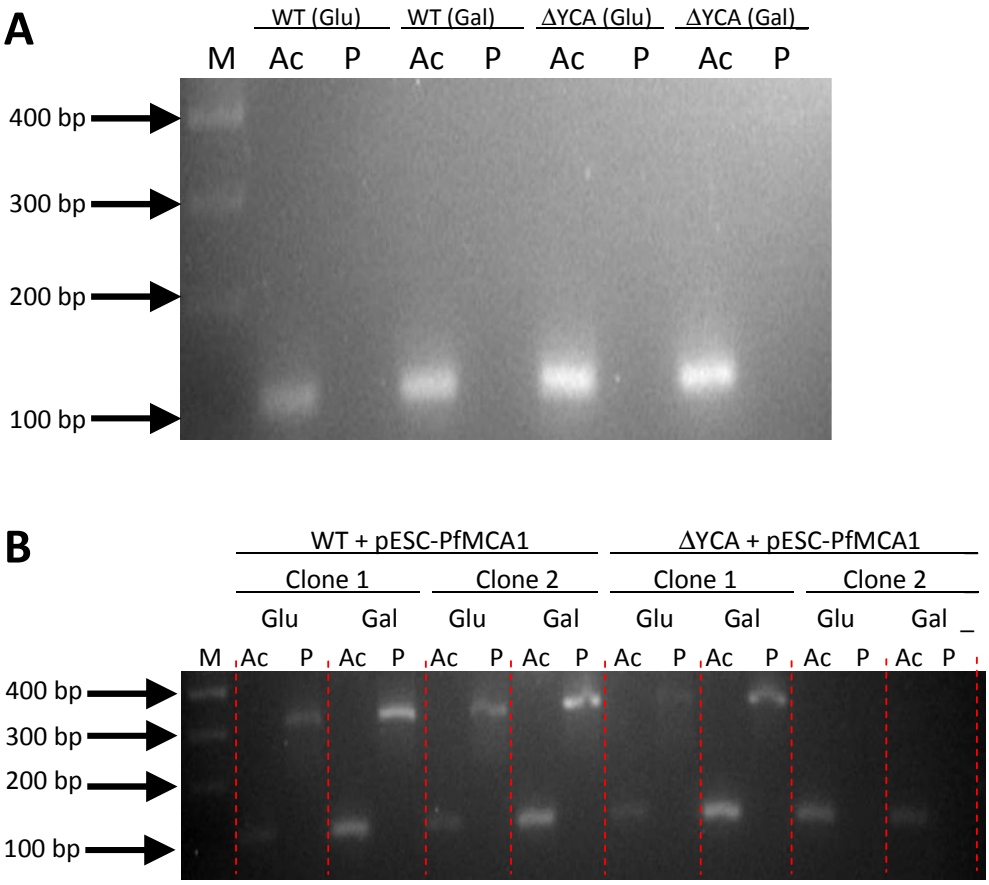

Figure 8. Reverse-transcriptase PCR of RNA isolated from WT & ΔYCA1 yeast transformed with PfMCA1.
S. cerevisiae actin-specific primers would give a 117 bp band, while the PfMCA1-specific primers would give a 310 bp band. A 2.5% (w/v) agarose gel was used for electrophoresis.
A. Gel shows the controls for the primers used for RT-PCR. Actin-specific primers (Ac) and PfMCA1-specific primers (P) were used to detect actin and PfMCA1 mRNA transcripts respectively. RNA was obtained from WT and ΔYCA yeast grown in both non-inducing (Glu) and inducing media (Gal). Actin mRNA is present in all samples, while no PfMCA1 mRNA was observed. Lane M: DNA ladder
B. RT-PCR was performed on several clones of PfMCA1-transformed yeast. The same actin-specific (Ac) and PfMCA1-specific (P) primers were used for detecting the presence of the respective mRNA transcripts in both WT and ΔYCA yeast transformed with the pESC-PfMCA1 plasmid, grown in both non-inducing (Glu) and inducing media (Gal). Lane M: DNA ladder.

RT-PCR performed on cDNA samples obtained from untransformed WT and ΔYCA1 yeast showed the expected band for actin, and the absence of any bands using PfMCA1-specific primers (Fig. 8A). No PfMCA1 mRNA transcripts were present in the yeast host strains, and any that were detected had to be due to the presence of the PfMCA1-pESC recombinant plasmid.

Indeed, cDNA obtained from PfMCA1-transformed WT and ΔYCA1 yeast cells showed positive bands when PfMCA1-specific primers were used. Interestingly, the PfMCA1 mRNA transcripts could be detected even in samples that were grown in non-inducing conditions (Fig. 8B).

Despite the presence of PfMCA1 transcripts in transformed yeast cells grown under inducing conditions, no protein products could be detected. This observation suggests that the protein has an extremely high rate of turnover, and it is rapidly degraded, perhaps as soon as it is made. Alternatively, the mRNA may be regulated in a manner such that even though transcripts were present, these transcripts were not further processed for translation.

3.6 Low complexity regions in PfMCA1

SEG analysis showed that 43%, almost half of the protein consists of low complexity regions. Low complexity regions are believed to form non-globular protein domans, and the strong presence of low complexity regions is not uncommon, although it is unique to *P. falciparum* proteins (Pizzi and Frontali, 2001). Low complexity regions exceeding 29% of the total protein primary structure often prevents successful heterologous expression (Birkholtz *et al*., 2008).

Low Complexity	Position	High Complexity
	1–88	MEKIYVKIYELSGLEDKDNFSCYIKIY WQNKKYKSCILQKNPYKFNEIFLLPID IKNNVKDEKNNILSIEVWSSGILNNNK IAYTFFE
ldhirrerissekinlidvvkkctlqi svhiinnnqdilfcnikdifgnnkndk eihdailkyggnerhiikelrkekeig qynniyfndyvnvlntdpsqnyiyndm pkitpnniynnmnndqtnhtylkapns lynnentiyssnvhystymnnsptykn snnmnhvtnmyasndlhnsnhfkphsn aystinydnnnyiypqnhtniyn	89–300	
	301–562	RASPGSDQTLYFSPCNQKKALLIGINY YGTKYELNGCTNDTLRMKDLLVTKYKF YDSSNNIVRLIDNEANPNYRPTRRNIL SALMWLTRDNKPGDILFFLFSGHGSQE KDHNHIEKDGYNESILPSDFETEGVII DDELHKYLIQPLNEGVKLIAVVDSCNS GSSIDLAYKYKLKSKKWKEDKNPFHVI CDVTQFSGCKDKEVSYEVNTGQIAPGG SLVTAMVQILKNNMNTPSIITYEYLLH NIHAHVKQHSNQTVTFMSS
qkfnmnrlfdfehiiknknnqlgqiin kyieknksknknklkhelknlff	563–612	
	613–613	F

Table 4. SEG output showing low complexity regions in PfMCA1.
Close to 50% of the PfMCA1 protein consists of regions of low complexity.

3.7 Expression of optimized PfMCA1 in *E. coli*

To see whether PfMCA1 could also be expressed using other protein expression systems, the optimized PfMCA1 coding sequence was cloned into an *E. coli* expression vector, pGEX. The pGEX vector allows for high level of inducible protein expression in *E.* coli as fusion proteins with the glutathione S-transferase (GST) enzyme at the C-terminus. The empty vector and the recombinant vector with PfMCA1 (PfMCA-pGEX) were used for transformation of *E. coli* strain BL21 (DE3).

Using anti-histidine antibodies, immunoblots using protein extracts from transformed *E. coli* cells did not show any positive bands (Fig. 9A). On the other hand, anti-GST antibodies revealed a whole range of positive protein bands (Fig. 9B). This was attributed to heavy background contamination, as bands were observed even in samples obtained under non-inducing conditions. The Coomassie Blue stain (Fig 9A) showed the expected GST protein at the 25 kDa band with samples transformed with the empty pGEX vector and grown under inducing conditions. However, no significant band was observed at the 100 kDa mark, which is the approximate combined size of PfMCA1 and the GST moiety.

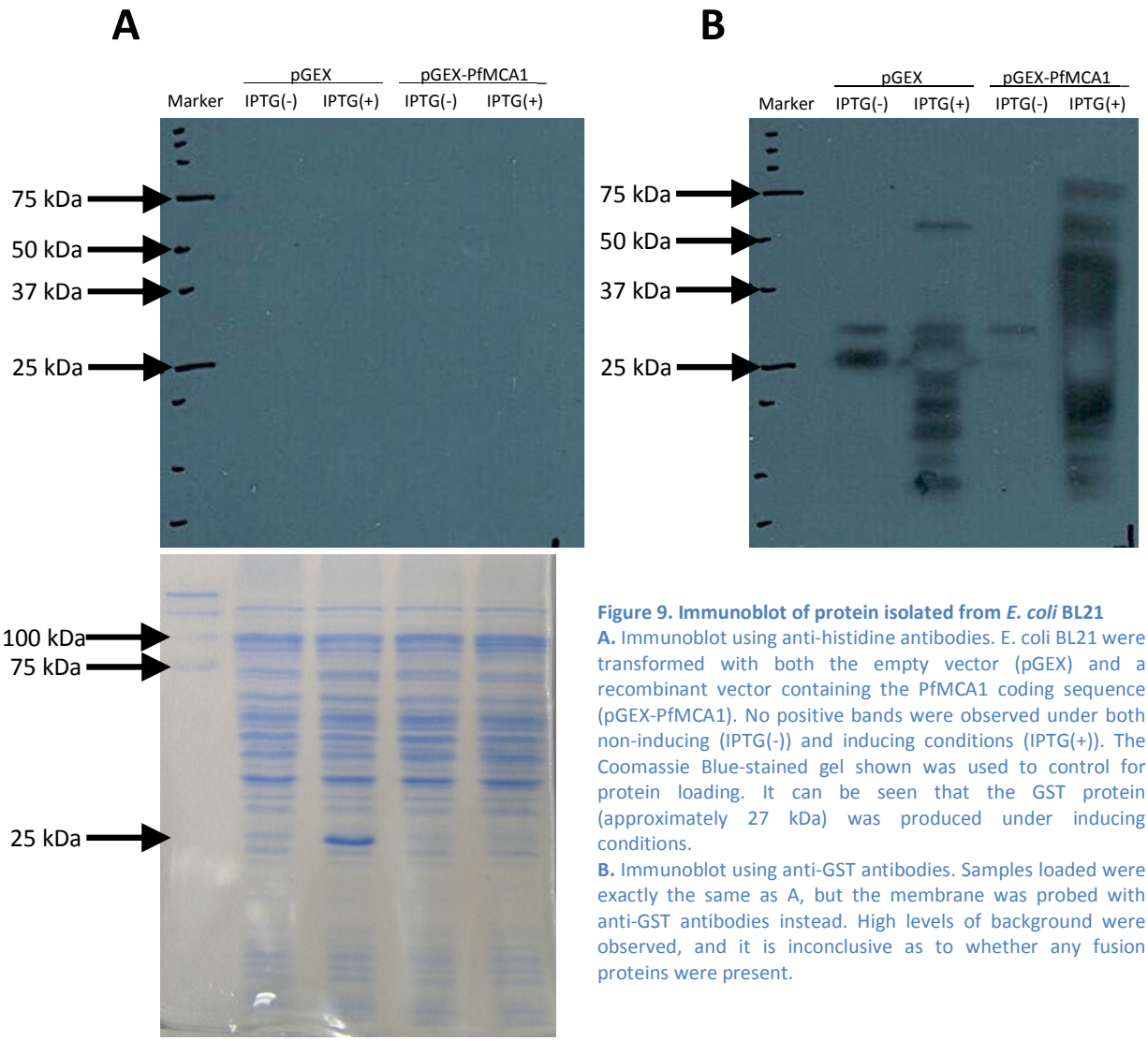

Figure 9. Immunoblot of protein isolated from *E. coli* BL21
A. Immunoblot using anti-histidine antibodies. E. coli BL21 were transformed with both the empty vector (pGEX) and a recombinant vector containing the PfMCA1 coding sequence (pGEX-PfMCA1). No positive bands were observed under both non-inducing (IPTG(-)) and inducing conditions (IPTG(+)). The Coomassie Blue-stained gel shown was used to control for protein loading. It can be seen that the GST protein (approximately 27 kDa) was produced under inducing conditions.
B. Immunoblot using anti-GST antibodies. Samples loaded were exactly the same as A, but the membrane was probed with anti-GST antibodies instead. High levels of background were observed, and it is inconclusive as to whether any fusion proteins were present.

3.8 Expression of optimized PfMCA1 in *T. brucei*

Attempts were made to express PfMCA1 in trypanosomes using *T. brucei* as the host. The expressed PfMCA1 protein would be tagged with yellow fluorescent protein (YFP), allowing visual confirmation of protein expression with a fluorescence microscope. Not unlike *Plasmodium*, trypanosomes are unicellular bloodborne parasitic protozoa, and they possess five metacaspases, one of which has been implicated in *S. cerevisiae* cell death (Szallies *et al*., 2002).

Transient expression of PfMCA1 had a small degree of success. The optimized sequence of PfMCA1 was used throughout these series of experiments on *T. brucei*. Yellow flourescent trypanosomes were observed in both healthy and dying cells (loss of characteristic cell morphology) (Fig. 10). The percentage of cells that successfully express PfMCA-YFP was also low – out of the 1×10^7 trypanosome cells that were electroporated, only an average of 10 cells were observed to be glowing yellow. A much larger area also had to be visually scanned before a fluorescent trypanosome could be located. In contrast, fluorescent trypanosomes could be immediately observed when they were transformed with the empty plasmid vector. Often, within the same field, several fluorescent trypanosomes could be seen, while only a single fluorescent trypanosome could be seen for those expressing PfMCA1. These observations suggest that the PfMCA1 protein has an extremely low level of expression in trypanosomes, and it may somehow be involved in trypanosomal cell death.

In an attempt to increase the expression level of PfMCA1 in trypanosomes, the PfMCA1 gene was stably-integrated into the genome of *T. brucei*. While cultures of transformed *T. brucei* could be grown in the presence of antibiotics, no viable clones could be isolated. An interesting observation is that under inducing conditions, growth of the transformed trypanosome culture was slower than that of a similar culture growing under non-inducing conditions, suggesting that even though there was no detectable expression of PfMCA, its expression has a negative impact on cell growth.

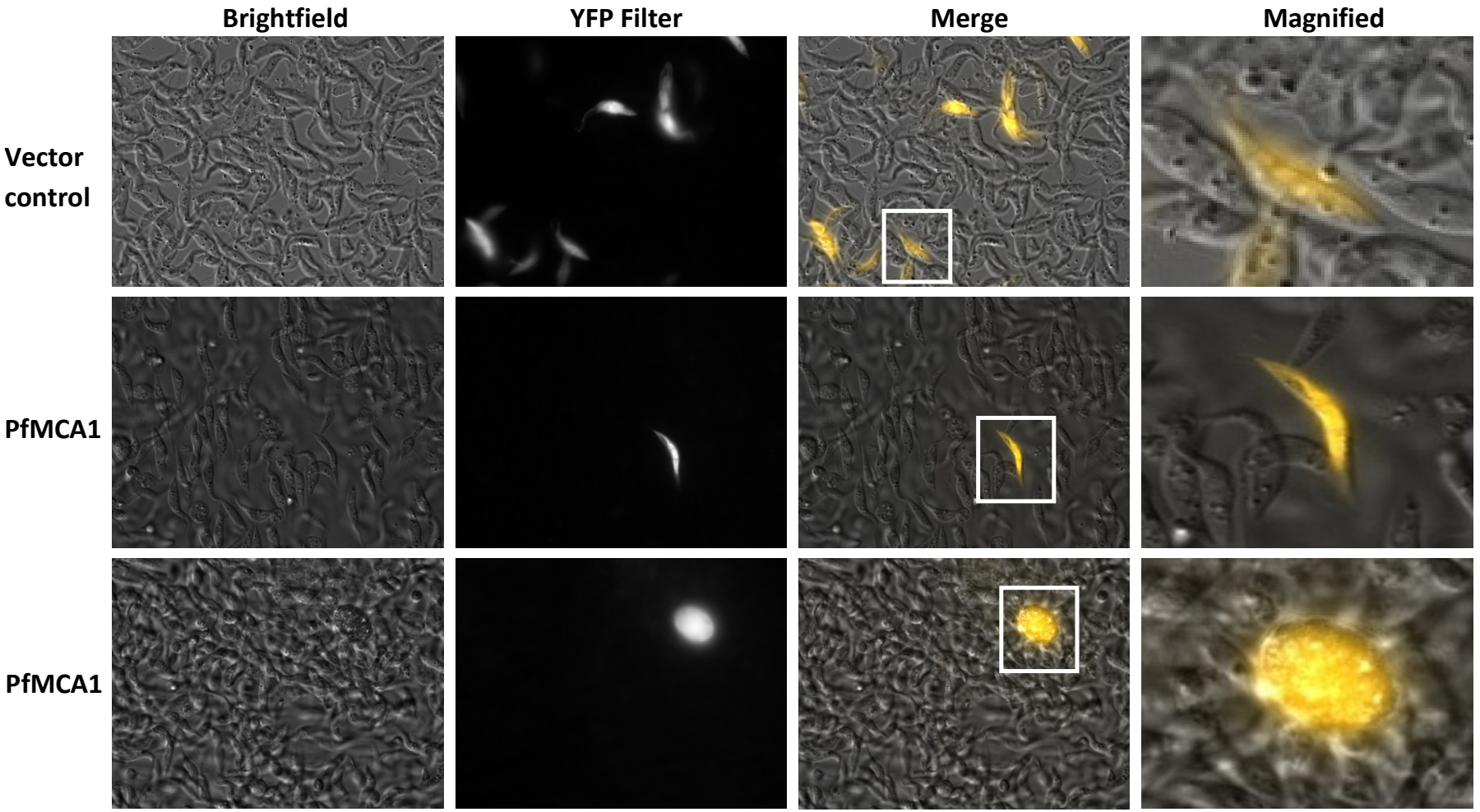

Figure 10. Expression of PfMCA1-YFP fusion proteins in *T. brucei*. PfMCA was cloned into a *T. brucei* expression plasmid vector, and transiently expressed. Trypanosomes transformed with an empty vector showed the expected yellow fluorescence. PfMCA1 could be expressed in trypanosomes, as evidenced by the same yellow fluorescence when the vector with the PfMCA1 gene was used for transformation. However, the degree of expression is significantly lower than that of the empty vector. In addition, expression of PfMCA1 could be found in healthy cells (middle row), as well as cells that are dying (bottom row). Dying cells tend to lose the characteristic shape of the trypansomes and exhibit a round morphology. Photos were taken with live, moving cells, hence in the composite images, the images may not match up exactly. Pictures are representative of 12 and 24 hours timepoints. Boxed areas have been magnified.

3.9 RNAi in *T. brucei*

A nucleotide sequence was designed to silence metacaspase 4 of *T. brucei* (TbMCA4), as a previous study has shown that it is involved in the cell death pathway of *S. cerevisiae* (Szallies *et al*., 2002). This was cloned into the p2T7 vector, and the recombinant plasmid was inserted into *T. brucei* cells via electroporation.

Successful clones were isolated, as described in section 2.4.7, and four clones were picked for reverse-transcriptase PCR, to ensure that the RNAi was successful (Fig. 11). The silencing effect is inducible, and is controlled by a Tet system. Presence of tetracycline in the culture medium would induce the production of the interfering RNA, leading to gene silencing.

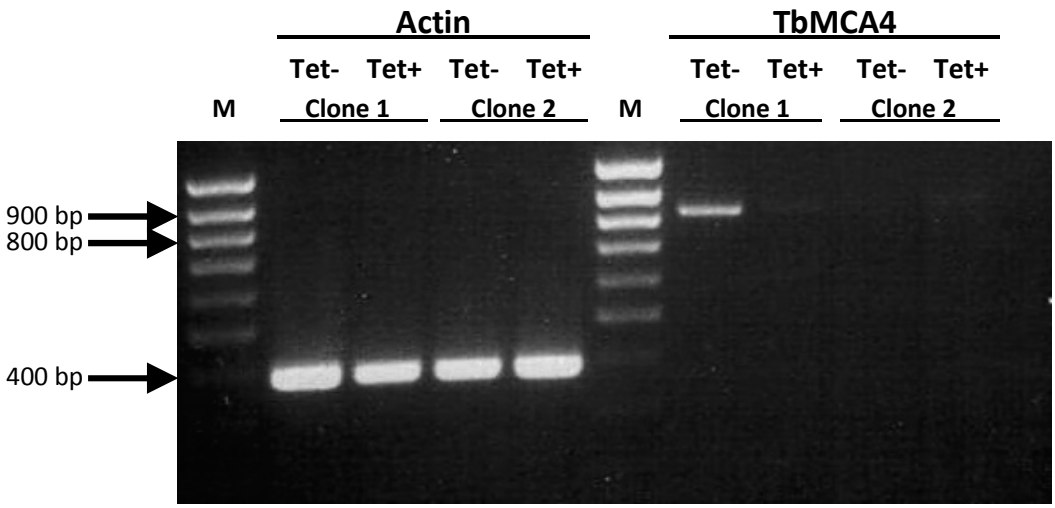

Figure 11. Reverse-transcriptase PCR of RNA extracted from *T. brucei* clones.
RNA was extracted from isolated *T. brucei* clones that had been successfully transformed with a TbMCA4 RNAi fragment. Isolated clones were grown under RNAi-non-inducing (Tet-) and inducing conditions (Tet+). Lanes 1 -4 used actin-specific primers for PCR, and lanes 5-8 used TbMCA4-specific primers. Lanes 1, 2, 5 and 6 represent RNA isolated from the same clone, while lanes 3, 4, 7 and 8 represents a second clone. In total, 4 clones were isolated, but the remaining clones showed the same results as the second clone. Lane M are loaded with DNA ladder. Both lanes are identical.

Of the four clones that were isolated, only one showed a presence of TbMCA4 mRNA transcripts (819 base-pairs) before RNAi induction, and demonstrated a significant decrease after (Fig. 11, lanes 5 & 6). The other three clones did not show any significant levels of TbMCA4 mRNA under both conditions, even though the level of actin transcripts (390 base-pairs) remained constant throughout for the four clones (Fig. 11, lanes 7 & 8).

3.10 Concanavalin A treatment assay

The TbMCA4-silenced clone previously isolated was used to determine the effect of TbMCA4-knockdown via treatment with ConA. Initial results using 50 μg/ml of ConA showed a lethal rate of almost 100%. No viable trypanosomes were observed, and those that did survive displayed sluggish movement. For practical purposes, it was decided the determination of the ideal concentration of ConA would begin with smaller concentrations.

Preliminary results showed that at 1.0 μg/ml of ConA, there were very little differences between the various conditions (Fig. 12A). Owing to significant variation in the results, it would be difficult to draw any significant conclusions.

A cleaner picture was obtained using 2.5 μg/ml of ConA (Fig. 12B). For ConA-treated cultures, TbMCA4-silencing (Fig. 12B, Tet+ ConA+) led to a greater survival rate, compared to the non-TbMCA4-silenced cultures (Fig. 12B, Tet- ConA+). The silenced cultures also experience a much more gradual increase and decrease in cell density (Fig. 12B, Tet+). TbMCA4-silencing probably comes with a metabolic cost, resulting in a much slower growth rate, but such cells demonstrate a greater resistance to cell death caused by nutrient depletion and senescence. In contrast, cultures still expressing TbMCA4 showed a steeper increase after 48 hours, possibly due to depletion of ConA in the culture medium (Fig. 12B, Tet-). This decrease in ConA concentration would allow any survivors to rapidly divide, since the selection pressure has dropped. Once the nutrients in the medium had been used up, the cells die at a faster rate as they are less resistant to cell death.

In cultures treated with PBS, the vehicular control for ConA, TbMCA4-silenced cells also show a much slower growth rate, but have a greater resistance to cell death due to senescence (Fig.12B, Tet+ ConA-). In contrast, the non-silenced cultures grow and die more rapidly, suggesting that TbMCA4 might be involved in cell homeostasis. (Fig.12B, Tet- ConA-)

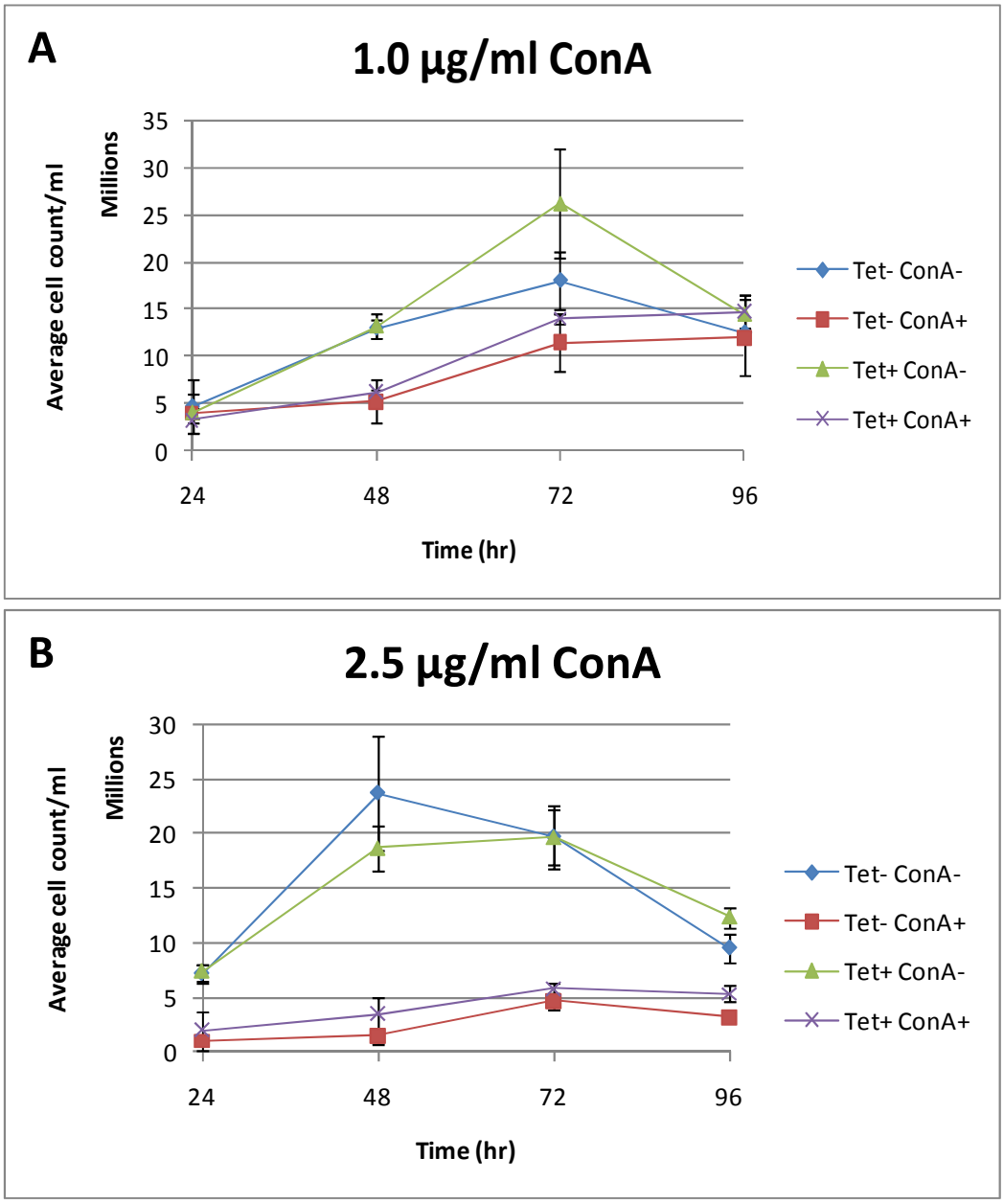

Figure 12. Effect of concanavalin A on TbMCA4-knockdown *T. brucei* cells.

T. brucei cells were treated with or without tetracycline, and with or without ConA. The assay was performed using 1.0 µg/ml (A) and 2.5 µg/ml ConA (B). Cells were counted visually every 24 hours for 96 hours. The assay was performed in triplicate, and an average of the three samples was used to obtain the value at each timepoint. Each timepoint using 1.0 µg/ml ConA represents an average of two readings. The third reading could not be used as the samples were not viable due to an unknown cause. Error bars represent the standard error. Raw data and calculations can be found in the appendix.

For the same time-point, pairs of data were compared, e.g. Tet-ConA- and Tet-ConA+, using the unpaired, two-tailed student's t-test, assuming unequal variance. For the values obtained using 1.0 µg/ml of ConA, the presence of ConA were not statistically significant (p-value>0.05). However, increasing the ConA to 2.5 µg/ml significantly decreased the viability of the cells (p-value<0.05). Calculations can be found in the appendix.

3.11 Site-directed mutagenesis of PfMCA1

The critical cysteine and histidine residues of the catalytic dyad have been identified, and to find out if these residues were important for enzymatic activity as predicted, they were mutated to alanine residues. The enzymatic activity of PfMCA1 may have an effect on the ability to express PfMCA1 heterologously, as the activity of PfMCA1 could be detrimental to the cell expressing it, resulting in low expression levels.

The full length optimized PfMCA1 gene sequence containing the cysteine-to-alanine mutation was cloned in the yeast plasmid vector pESC, and the DNA sequence was verified by DNA sequencing. Subsequent expression of the mutated protein in WT or ΔYCA1 yeast was not successful, despite repeated attempts.

3.12 Expression of PfMCA1 protein domains

Protein structural data on PfMCA1 retrieved from PlasmoDB (http://plasmodb.org/plasmo/home.jsp; Gene ID PF13_0289) identified amino acids 318 to 551 (amino acids 324 to 557 in the optimized coding sequence) as a conserved C14-peptidase caspase domain. BLASTing the protein sequence of PfMCA1 against a database of conserved protein domains showed the same result.

A set of PCR primers were designed to express the PfMCA1 protein from amino acids 1 to 317 (non-catalytic domain), and another set to express the rest of the PfMCA1 protein from amino acids 318 (catalytic domain) onwards. The optimized coding sequence of PfMCA1 was used, and the hexahistidine tag at the N-terminus was removed from the sequence. These two fragments were cloned into the pESC yeast plasmid vector, and expressed in both WT and ΔYCA1 yeast cells.

The results of the expresson of the catalytic domain were similar to the expression of the full-length protein. No expression could be detected with immunoblotting, even with repeated attempts.

In contrast, expression of the non-catalytic domain could be observed. A predicted protein band approximately 35 kDa in size was could be detected on the immunoblot (Fig. 13). While expression of the non-catalytic domain was strong in the WT yeast cells, with a thick band being observed, expression in ΔYCA1 yeast cells was relatively weak.

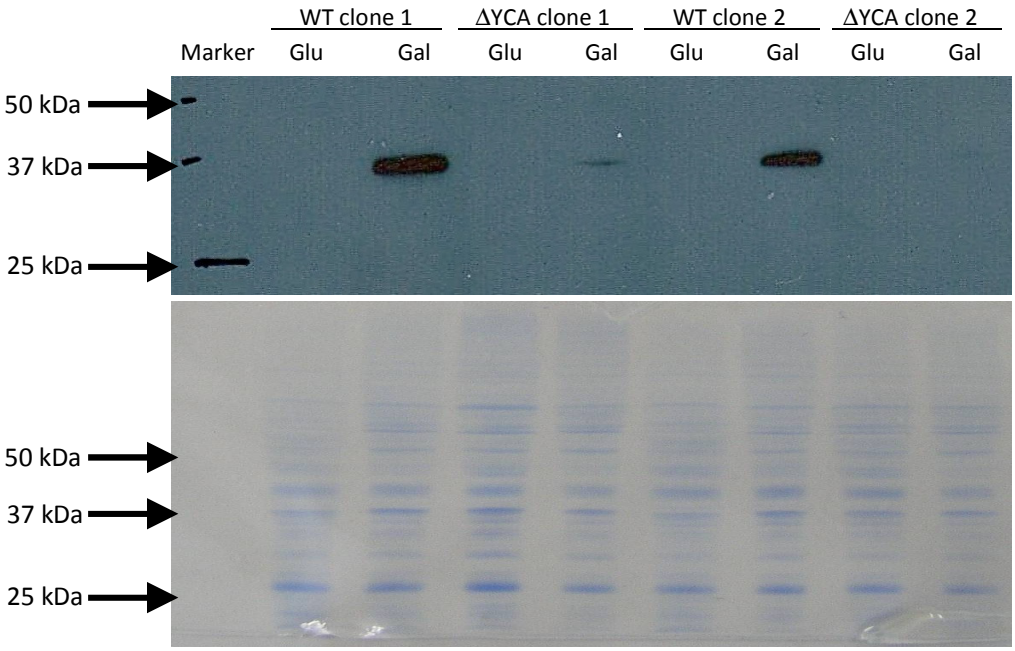

Figure 13. Expression of the protein domains of PfMCA1. The catalytic domain of PfMCA1 (amino acids 318 to 613) could not be expressed (results not shown). On the other hand, the expression of the non-catalytic domain of PfMCA1 (amino acids 1 to 317) could be successfully detected. A protein band corresponding to the predicted size (35 kDa) of the non-catalytic domain could be seen in both transformed WT and ΔYCA1 yeast cells. It is interesting to note that the expression levels of the non-catalytic domain varied between the two yeast strains. The expression was strong in transformed WT yeasts, but it was extremely weak in transformed ΔYCA1 yeasts, almost undetectable. Two clones were isolated and tested for protein expression. The Coomassie Blue stain (shown below the immunoblot) was used as protein loading controls.

4. DISCUSSION

Previously, programmed cell death has been extensively studied only in multicellular systems, as it was first observed in metazoans, and it seemed only natural to assume that any organism that would benefit from such a cell suicide program would be a multicellular one. Afterall, it made no altruistic sense for unicellular organisms.

However, in the last decade or so, it was discovered that PCD is not exclusive to metazoans, but that it is present as well in unicellular eukaryotes. This has sparked great interest in the subject, and several studies have been conducted to study this phenomenon and its similarities to classical PCD, including its mediators and pathways.

Many studies have found characteristics of PCD in *Plasmodium*, induced by a variety of agents, such as febrile temperatures (Oakley *et al*., 2007), reactive oxygen species (Kumar *et al*., 2007), platelets (McMorran *et al*., 2009) and drugs (Meslin *et al*., 2007). However, this has been disputed by other studies. Al-Olayan *et al.*, (2002) have shown that hallmarks of PCD, such as chromatin condensation and phosphatidylserine exposure, were present in malarial parasites that were invading the mosquito midgut. However, these results could not be replicated when attempted by Le Chat *et al*., (2007). Other studies also failed to detect signs of PCD in malaria parasites (Deponte and Becker, 2004; Nyakeriga *et al*., 2006; Totino *et al*., 2008).

Regardless, a class of caspase-like proteins, the metacaspases, have the potential to act as mediators in the PCD pathway, and this was first demonstrated by Madeo *et al*., (2002), when they showed that a knockout of the *S. cerevisiae* metacaspase YCA1 was more resistant to cell death-inducing stimuli. It was also fortuitous that this was done in yeast, as it gave other researchers a model eukaryotic system to investigate metacaspases from other organisms.

Of the three metacaspases that have been identified in *P. falciparum*, PfMCA1 bears the greatest similarity to YCA1, and it has been postulated to possess a similar function. To

date, only one study has attempted to study the characteristics of PfMCA1 (Meslin *et al*., 2007). However, that particular study has only managed to demonstrate the successful heterologous expression of PfMCA1; it still remains to be seen if it is functionally similar to YCA1, or has other biological functions.

There were several obstacles during the course of this study. For example, PfMCA1 required several rounds of optimization before it could be successfully amplified, and coupled with operator unfamiliarity with the techniques involved, an inordinate amount of time was spent cloning the PfMCA coding sequence into yeast expression vectors.

4.1 Molecular cloning

Initially, genomic DNA was isolated from a culture of *P. falciparum* strain 3D7 using the QIAamp DNA Blood Mini Kit (QIAgen). The kit utilises a silica-gel membrane to selectively bind DNA while allowing other cellular debris and contaminants to be washed away. Pure DNA is then released from the membrane when it is incubated with either water or the elution buffer provided with the kit.

During normal development, *Plasmodium* parasites break down proteins in erythrocytes to obtain amino acids to support their growth. As haemoglobin makes up a significant proportion of the total protein content of erythrocytes, *Plasmodium* parasites have to find a way to detoxify the toxic haem residues that are produced during haemoglobin degradation. This is achieved by converting the haem residues to haemozoin crystals (Kirchgatter and Del Portillo, 2005).

This poses a unique problem with the isolation of genomic DNA using the QIAamp kit. A significant amount of the haemozoin pigment was retained on the silica-gel membrane, leading to poor yield of DNA after elution, possibly due to occlusion of the silica-gel membrane, which resulted in a lower available surface area for DNA binding. The first extraction only yielded 2.6 ng/µl of DNA; subsequent repeats of the isolation protocol only slightly improved the yield to approximately 15 ng/µl. Using higher quantities of parasites did

not improve the yield significantly, suggesting that the rate-limiting factor was the adsorption of genomic DNA to the silica-gel membrane. Despite the low yield, the quality of the isolated genomic DNA was within acceptable parameters – spectrometry readings using the NanoDrop machine showed the DNA to be free of any contaminating proteins or carbohydrates.

The genomic DNA was used for amplifying the *Plasmodium* metacaspase gene, PfMCA1, which is 1,842 base-pairs in length, using the primers 5'PfMCA-EcoRI and 3'PfMCA-SalI. In an attempt to determine the optimum annealing temperature, gradient PCR was performed, ranging from 30-60^{o}C. Several attempts were made to optimize the annealing temperature, but only faint bands were observed throughout the entire range of annealing temperatures. While these faint bands were of the expected size, there was significant smearing and unspecific products due to primer-dimer formation.

In order to obtain genomic DNA of higher quality for PCR amplification, the phenol-chloroform method was used instead. Due to the organic nature of the reagents, DNA is cleanly partitioned in the aqueous phase, allowing a much greater yield of genomic DNA, as compared to using a kit. Indeed, significantly higher amounts of DNA (approximately four times more) were obtained with the same amount of parasites, with typical yields ranging from 30 to 60 ng/µl.

Using this genomic DNA as a template, PCR conditions were optimized by varying the conditions one at a time. Previous gradient PCR runs indicated that the optimum annealing temperature for the PfMCA1 gene lies between 40^{o}C and 60^{o}C. Further investigation revealed the PCR yield reached a peak at a temperature of 51^{o}C. However, this wasn't sufficient for subsequent molecular cloning, as the band was still deemed too faint. Adjusting the Mg^{2+} concentration to 2.0 mM in the PCR reaction mix improved the intensity of the band significantly.

Compared to PfMCA1, amplification of the YCA1 gene was relatively straightforward. Genomic DNA isolated using the protocol of Harju *et al.*, (2004) was used for PCR amplification using the primers 5'YCA1-EcoRI and 3'YCA1-SalI. Sufficient

amounts of YCA1 PCR products were obtained with a Mg^{2+} concentration of 1.5 mM and an annealing temperature of 51°C, hence no further optimization was carried out.

The PfMCA1 and YCA1 PCR products were digested with the restriction enzymes EcoRI and SalI. The plasmid vector PactTHA423 was similarly digested. The digested products were then ligated together in various ratios, using the following formula

$$\text{Volume of insert (µl)} = \frac{\text{Amount of vector (ng)} \times \text{Size of insert (kbp)} \times \text{Ratio}}{\text{Size of vector (kbp)} \times \text{Concentration of insert (ng/µl)}}$$

where kbp refers to kilo base-pairs. However, initial attempts to transform *E. coli* strain DH5α cells proves unsuccessful. Very few, or no positive colonies were observed when the transformed cells were plated onto LB agar plates in the presence of ampicillin. Restriction enzyme analysis of the plasmids extracted from the positive clones did not reveal any successful clones.

Further investigation revealed that a high proportion of the digested plasmid vector underwent self-recircularization, resulting in an extremely low cloning efficiency. This was entirely unexpected as the plasmid vector was digested with two different restriction endonucleases to give incompatible ends, and as such, would prevent such self-recircularization from ever occuring in the first place. To prevent such recircularization and improve the cloning success rate, the plasmid vector was treated with Antarctic Phosphatase (New England Biolabs) to remove the phosphate group. The phosphate group is an important element of the ligation process, and its absence from the plasmid vector meant that any ligation reaction would require the phosphate group from an exogenous source. In this case, the phosphate group would come from the gene sequence to be inserted into the plasmid vector. The cloning efficiency improved significantly after treatment of the plasmid vector with phosphatase, and subsequent cloning efforts incorporated the phosphatase treatment.

Expression of PfMCA1 in yeast cells was unsuccessful, despite extensive troubleshooting. Initial DNA sequencing revealed several mutations in the coding sequence, which could have resulted in premature termination or nonsense codons. Taq polymerase has

a relatively low fidelity, having an error rate of 1 in 9,000, and a frameshift mutation rate of 1 in 41,000 (Tindall and Kunkel, 1998). Taq polymerase also does not possess any 3'→5' exonuclease proofreading capabilities (Lawyer *et al*., 1993), making it unable to correct any mistakes it has made during the replication process.

PfMCA1 is a long gene, 1,842 base-pairs in length, and the error rate of Taq polymerase means that approximately 1 in 5 copies will possess an error of some kind. If the mutations occur early in the PCR process, the amount of error-free products will be extremely small, due to the exponential amplification power of PCR. This prompted the change of DNA polymerase from Taq to a high fidelity polymerase system, significantly reducing the amount of mutations in the coding sequence. The new PCR system utilizes a polymerase mixture comprising of Taq and Tgo polymerases. The Tgo polymerase compensates for the lack of proofreading capability in Taq, increasing the fidelity of DNA replication by a factor of 3, and dramatically increasing the amount of error-free PCR products.

Interestingly, the amount of mutations/errors observed with YCA1 is much less than that observed for PfMCA1. Often, with Taq polymerase, PfMCA1 recombinant plasmids sent for sequencing results in all of them having an error of some kind, while YCA1 recombinant plasmids have few or almost no mutations at all. This may be in part due to YCA1 being of a smaller size (1,296 base-pairs), but it seems that the high (A+T)-content of PfMCA1 gene plays a more significant role. A-T base-pairs consists of two hydrogen bonds, and with a high (A+T)-content, much lower annealing temperatures are required, leading to non-specific priming and misannealing of primers (Chevet *et al*., 1995). Continuous stretches of adenosines and thymidines in the coding sequence could also also have led to copy errors in the PCR products (Mehlin *et al*., 2006).

4.2 PfMCA1 expression in *S. cerevisiae*

Plasmodial proteins are notoriously difficult to express heterologously in host expression systems, and a variety of teachniques have to be employed for successful

expression (Birkholtz *et al*., 2008). While there have been successful cases of expressions (Meslin *et al*., 2007; Salas *et al*., 1995; Withers-Martinez *et al*., 1999; Yadava and Ockenhouse, 2003; Zhang *et al*., 2002), difficulties with expression have hindered detailed biochemical and functional analyses (Birkholtz *et al*., 2008; Salas *et al*., 1995)s. Early attempts at PfMCA1 expression in yeast did not yield positive results, and this remained so with both constitutive and inducible promotors.

Several reasons have been postulated to account for the difficulties that were encountered in attempting to express plasmodial proteins. As mentioned earlier, the high (A+T)-content of the plasmodial genomes, as high as 80% for *P. falciparum*, have been blamed for the low level of expression. Long repeats of adenosine and thymidine bases make it difficult for host organisms to replicate and translate faithfully. However, studies have shown that the (A+T)-content is not a significant factor in protein expression, at least in *E. coli* (Mehlin *et al*., 2006; Vedadi *et al*., 2007).

Instead, the high (A+T)-content translates to a codon bias that greatly hinders protein expression. (A+T)-biased codons, such as lysine and asparagine, means that these amino acids are used up relatively quickly (Vedadi *et al*., 2007), and the prevalance of rare codons, e.g. AGA and AGG in *E. coli*, imposes a metabolic strain on the host (Birkholtz *et al*., 2008). This can trigger a phenomenon known as ribosomal stalling, where the lack of certain amino acids causes peptide elongation to stop and terminate (Birkholtz *et al*., 2008), leading to truncated proteins and low expression levels.

Another reason put forward for the low expression of plasmodial proteins suggests that plasmodial proteins are toxic to the host organism. This results in a lower growth rate of the host cells, and the complete lack of protein expression (Birkholtz *et al*., 2008; Cinquin *et al*., 2001; Mehlin *et al*., 2006). It is not hard to imagine that the cell would prevent the synthesis of proteins that it deems toxic to itself.

In order to successfully express PfMCA1 in yeast, two important problems had to be overcome, that of tRNA exhaustion, and preventing long stretches of adenosines from being recognized as polyadenation or transcription termination signals. The protein sequence of

PfMCA1 was reverse-translated into a DNA sequence which will suit the codon bias of the host organism, in this case, *S. cerevisiae*. Synthetic genes can be manufactured by a sequential series of PCR, generating desired fragments via careful design of primers and subsequent assembly of the entire coding sequence using these fragements. This approach has allowed the successful expression of the *P. falciparum* subtilisin-like protease *pfsub1* in *Pichia pastoris*, an alternative yeast expression system (Withers-Martinez, 1999).

A caveat to this expression strategy is that successful expression is not always guaranteed. Not all synthetic genes could be expressed, and even for those that were, some ended up being sequestered in inclusion bodies (Mehlin *et al*., 2006). A study that used *E. coli* as an expression system revealed that codon bias was not a significant factor in the expression level of individual genes. Instead, the stability and free energy of the mRNA, i.e its secondary structure, plays a more critical role (Kudla *et al*., 2009).

Even within the yeast cell, there are several things that could go wrong. Protein production can be too efficient, leading to a high concentration of proteins in the endoplasmic reticulum. This can signal the yeast to start the degradation of such aggregated proteins via the ubiquitination pathway (Mattanovich *et al*., 2004). Yeast proteins that are responsible for ensuring correct folding and disuphide bond formation could also differ from those in *P. falciparum*, resulting in an aberrant form (Romanos *et al*., 1992).

While there are many considerations that had to be taken into account, the decision was taken to synthesize a codon-optimized PfMCA1 coding sequence suitable for expression in *S. cerevisiae*. It would have been impossible to predict with absolute certainty whether such a synthetic gene could be successfully expressed – the only way to know for sure would be to actually clone the gene and test for expression.

Disappointingly, no expression could be detected with the synthetic gene. The central dogma of molecular biology states that in order to obtain protein from DNA, DNA has to be first transcribed to RNA, followed by a translation from RNA to protein (Crick, 1970). The RNA of various yeast transformants was isolated, and using PfMCA1-specific primers (and actin-specific primers as controls) for RT-PCR, it was discovered that PfMCA1 transcripts

were present. While this would not be surprising in yeast transformants grown under inducing conditions, PfMCA1 mRNA transcripts were also present under non-inducing conditions, suggesting that at the very least, there was basal transcription of the gene present. This could be possibly caused by the leaky transcription of the HIS3 auxotrophic selection gene (Pratt *et al*., 2007; Tian *et al*., 2001) and GAL10 promotor (Ruhela *et al*., 2004), resulting in a background level of PfMCA1 transcripts.

Evidence suggests that the yeast host cells do not tolerate the presence of the PfMCA1 protein very well, resulting in a lack of expression. Since expression in yeast has proven fruitless, other alternative expression systems were considered.

4.3 PfMCA1 expression in *E. coli*

E. coli is a commonly used host expression system, as the bacteria itself grows relatively quickly, and it is cheap, easy to use and genetically manipulate. The synthetic version of PfMCA1 was cloned into a pGEX vector, and the recombinant plasmid was used for transformation of *E. coli* strain BL21 (DE3). This strain of *E. coli* was chosen as the bacterial host for expression as it is protease-deficient, allowing for a greater degree of protein stability (Studier *et al*., 1990). To minimize the amount of protein being sequestered in inclusion bodies, bacterial cultures were incubated at room temperature. This would reduce the rate of protein synthesis, and would hopefully allow soluble expression.

The recombinant PfMCA1 protein would be expressed with a hexahistidine tag at the N-terminus, and a GST-moiety tag at the C-terminus. Unfortunately, no expression could be detected with anti-histidine antibodies. Using anti-GST antibodies resulted in an immunoblot with high levels of background, making it impossible to reach any significant conclusions. The Coomassie Blue stain stained gel, however, showed clearly that no PfMCA1 protein had been expressed. In bacteria that was transformed with the empty pGEX vector, those were grown under inducing conditions had an obvious band approximately 25 kDa in size, as opposed to none in bacteria without induction. This band corresponds to the size of the GST

moiety (27 kDa), which would be the only protein that would be heterologously expressed with an empty pGEX vector.

In contrast, there was no difference in band intensity between non-induced and induced cultures of bacteria transformed with the synthetic PfMCA1 gene. If PfMCA1 was indeed expressed, a band approximately 98 kDa in size, which is the total weight of the PfMCA1 protein and GST moiety, would be observed in bacteria grown with induction. It is possible that even if an increase in band intensity was not observed, it could still be detected with an immunoblot. However, the immunoblot results suggest otherwise, that no PfMCA1 protein had been expressed.

In *E. coli*, factors that prevent the soluble expression of plasmodial proteins include molecular weights of 56 kDa and beyond, greater protein disorder, isoelectric points of 6 and above, lack of homology to *E. coli* proteins, regions of low complexity (>29%) and plasmodium-specific protein motifs (Birkholtz *et al*., 2008). Examination of chromosomes 2 and 3 of *P. falciparum* revealed that a high percentage of the proteins coded for within those chromosomes contain regions of low complexity (Pizzi and Frontali, 2001), and it stands to reason that this is characteristic of the entire *P. falciparum* proteome (Gardner *et al*., 2002). The abundance of low complexity regions have been implicated as a mechanism for the malarial parasite to avoid host immune responses via antigenic variability (Dodin and Levoir, 2005).

Regions of low complexity can be determined bioinfomatically using SEG (Wootton and Federhen, 1996). SEG analysis showed that almost 43% of the protein consists of low complexity regions. Furthermore, PfMCA1 has a predicted size of 71.7 kDa and a predicted pI of 8.7 (PlasmoDB), and together, these factors would pose a formidable obstacle, and would certainly account for the lack of heterologous expression in *E. coli*.

4.4 PfMCA1 expression in *T. brucei*

Other bloodborne unicellular protozoan parasites possessing metacaspases include *Trypanosoma* and *Leishmania*. Five metacaspases have been identified in metacaspases, of which metacaspases 2, 3 and 5 have associated with the endosome pathway (Helms *et al*., 2006). Metacaspase 4 have been implicated in cell death, as heterologous expression induced respiratory deficiency in *S. cerevisiae* (Szallies *et al*., 2002). The role of metacaspase 1 remains unknown at this point in time.

T. brucei has previously been used as an expression host (Gannavaram *et al*., 2008), and are easy to cultivate and genetically manipulate. Transient expression of PfMCA1 tagged to YFP at the C-terminus in trypansomes did reveal successful expression, but the level of expression is extremely low compared to a control performed with an empty vector. In a 10 µl volume of control culture, glowing trypanosomes were plentiful and could be detected easily. In contrast, an average of ten positive trypansomes could be detected in the same 10 µl volume of culture that was transformed with PfMCA1. It is noteworthy that PfMCA1 expression could be detected in both healthy and dying trypanosomes, suggesting that PfMCA1 is somehow involved in the cell death pathway. Coupled with the low expression level, it hints that the inability to express PfMCA1 may be a result of the protein itself.

The yellow fluorescence was observed throughout the entire cell, and was not compartmentalized to any particular organelle, suggesting that PfMCA1-YFP is distributed in the cytoplasm. In contrast, heterologous expression of TbMCA4-GFP fusion proteins in *S. cerevisiae* demonstrated a nuclear localization. Similarly, YCA1-GFP fusion proteins also localized in the nucleus. However, the same YCA1-GFP protein could also be observed throughout the entire cell (Szallies *et al*., 2002, suggesting that YCA1 is involved in a variety of biological processes before and during cell death, but the nucleus is where most of its effects are felt. On the other hand, the effect of TbMCA4 seems to be localized to the nucleus. It seems that not unlike YCA1, PfMCA1 exerts its effects globally, but this result is presumptive at best without more concrete evidence.

Concurrent with the effort to express PfMCA1 in *T. brucei*, a system was being set up in *T. brucei* to investigate the effect of PfMCA1 on trypanosome cell death. It was decided that metacaspase 4 of *T. brucei* (TbMCA4) would be silenced, and a phenotypic rescue would be attempted with PfMCA1. Concanavalin A (ConA) have been shown to kill procyclic *T. brucei* (Acosta-Serrano *et al*., 2000), and it appears to do so with apoptotic characteristics (Welburn *et al*., 1996). Treatment of *T. brucei* cells with ConA causes the cells to lose their characteristic morphology, and become round. Treated cells will also tend to agglutinate. These observations are in agreement with what was previously described by Welburn *et al* (1996).

A clone was previously isolated that demonstrated RNAi silencing of TbMCA4. Preliminary data indicates that the TbMCA4-silenced culture was more resistant to cell death induced by ConA, as compared to a culture that did not have the TbMCA4 gene silenced. Silenced cultures also tend to have more motile trypanosomes that retained their characteristic morphology and less agglutination, as compared to the non-silenced cultures. However, the cultures recovered if left overnight, suggesting that the concentration of ConA used (2.5 µg/ml) might have been too little to last a prolonged period of time. Welburn *et al.* used 50 µg/ml ConA, which in our hands, caused a massive dying of cells, making it impossible to obtain any meaningful results.

These results indicate that TbMCA4 could have a possible role in the ConA-induced cell death pathway of *T. brucei*. However, as PfMCA1 did not express very well in trypanosomes, no further optimization of the ConA assay was carried out. The data does suggest that with further optimization of the conditions, this assay could prove to be useful for investigation of PCD in trypanosomes. Nevertheless, it should be noted that the presence of other metacaspases in *T. brucei* pose a risk of redundancy. Even though metacaspases 2, 3 and 5 have not been implicated in trypanosomal cell death, the role of metacaspase 1 has not been fully elucidated, and it is possible that any combination of these metacaspases might be able to assume the functions of TbMCA4.

4.5 Over-expression of YCA1

S. cerevisiae actin could be successfully over-expressed under the appropriate conditions, indicating that the expression system employed could not have prevented PfMCA1 expression. This suggested that a factor intrinsic to PfMCA1 is responsible for the inabilty to detect PfMCA1 expression. Curiously, neither could YCA1 be expressed as well, even though previous studies have successfully done so (Bettiga *et al*., 2004; Madeo *et al*., 2002; Watanabe and Lam 2005). Instead of using genomic DNA as the source, the YCA1 coding sequence was amplified from previously-isolated RNA. Using this fragment, recombinant YCA1 could be successfully detected. This is enigmatic, as the DNA sequences are virtually identical, as evidenced by DNA sequencing.

The exact mechanism of YCA1 processing is unknown, but it has been postulated to be similar to that of initiator caspases (Madeo *et al*., 2002; Watanabe and Lam 2005). Initiator caspases are present in the cell as inactive zymogens, which are able to undergo self-activation via autocatalysis. Overexpressed YCA1 was cleaved in transformed WT cells, while there was no such processing observed in transformed ΔYCA1 cells. This pattern of YCA1 processing certainly suggests an autocatalytic mechanism.

Without any form of cell signalling to initiate the processing, autocatalysis could only occur if the concentration of YCA1 in the cell reached a certain critical threshold level. In WT yeast cells, there already exists an endogenous pool of YCA1 molecules. It is therefore not inconceivable that upon induction of YCA1 expression, the concentration of YCA1 in the cell would reach a level sufficiently high enough to initiate autocatalysis, leading to the cleavage pattern observed in the immunoblot. In contrast, ΔYCA1 yeast cells lack any form of endogenous YCA1, and even with the overexpression, the level of YCA1 was not sufficiently high to cause autocatalysis. Thus, any YCA1 proteins heterologously expressed in ΔYCA1 yeast cells remained unprocessed, and were observed as full length proteins.

4.6 Amplification of PfMCA1 from RNA

As it seems that the coding sequence obtained from mRNA seemed to be much better suited for expression, mRNA was isolated from *P. falciparum*, in the hopes that a PfMCA1 mRNA transcript might be amplified for gene expression. Despite repeated attempts, no full-length mRNA transcript could be obtained.

Using PfMCA1 sequencing primers, it was possible to amplify segments of PfMCA1 from cDNA (results not shown). The largest fragment that was obtained spanned a region starting from nucleotide positions 519 to 1227 of the PfMCA1 coding sequence, a region that lies within the postulated non-catalytic domain (the catalytic domain starts from nucleotide position1651). This fragment was obtained using the primers PfMCA-pESC-fw-4893 and PfMCA-pESC-rv-5601. No PCR product was obtained when using sequencing primers which amplified outside of this region.

In contrast, other studies utilising microarray analyses and RT-PCR showed that the PfMCA1 gene was actively transcribed (Wu *et al*., 2003), and that it could be amplified from a cDNA library (Deponte and Becker, 2004). Certainly, evidence suggests that amplification of the full-length transcript should be possible, yet it is curious that that is not the case in our hands.

Laboratory culture represent a stringent set of conditions to which the organism being cultured has to adapt to. *In vitro* conditions are optimized for the organism's growth and reproduction, and differ greatly from conditions in its natural environment. For example, *P. falciparum* demonstrated down-regulation of PfEMP1 when field isolates were cultivated *in vitro* (Peters *et al*., 2007). Presumably, the lack of an immune response under *in vitro* conditions would signal the parasites that a strong *var* response is no longer required for immune evasion, and resources could be better utilized elsewhere. It is possible that extended culture of a laboratory-attenuated strain such as 3D7 might have led to post-transcriptional modifications of PfMCA1 mRNA to better suit its purposes, preventing successful amplification from RNA. Internal mutations leading to primer annealing failure are unlikely, as the PfMCA1 genomic sequence is identical to the one stored on the PlasmoDB database.

Another possibility is that the predicted gene sequence is incorrect, leading to inaccurate intron/exon predictions (PfMCA1 is predicted to have no introns), and other misinformation (Lu *et al*., 2007)

4.7 Expression of PfMCA1 variants

All classical caspases possess the same three-dimensional characteristics, quaternary arrangement and catalytic mechanism (Grütter, 2000). The imidazole group of the histidine residue reacts with the sidechain of the cysteine residue, activating it via polarization. Both the activated cysteine residue and the histidine residue cooperate to proteolytically cleave the substrate molecule. Metacaspases have been predicted to possess the same two residues, and it is possible that their mechanism is largely similar to caspases.

It is possible that the activity of PfMCA1 may be preventing any expression. To test that hypothesis, the cysteine residue of the catalytic dyad in PfMCA1 was mutated to an alanine residue via site-directed mutagenesis. The cysteine residue was chosen as the first amino acid to be mutated as previous studies have shown that the cysteine residue plays a more important role in the enzymatic activity of metacaspases, and hence their functional role in programmed cell death, as compared to the histidine residue (Gonzáles *et al*., 2007; Szallies *et al*., 2002; Watanabe and Lam, 2005).

Disappointingly, no PfMCA1 expression could be detected. Originally, it was hoped that other site-directed mutants, namely one where only the critical histidine residue had been similarly mutated to alanine, and a dual-mutation where both residues were mutated, could be generated, and these mutants could subsequently be tested for the individual and additive effects of such mutations on protein expression. However, previous efforts at troubleshooting and repeating protocols meant there was little time left to examine whether these mutants would have been successfully expressed.

In addition to the site-directed mutants, the catalytic and non-catalytic domains were also expressed in *S. cerevisiae*. No expression of the PfMCA1 catalytic domain could be

detected, but expression of the non-catalytic domain was successful. These results implied that the catalytic domain, and by extension its catalytic activity, is toxic to the yeast cells, or any other host organism. In response, the host organism either degrades the protein, or only allows an undetectable low-level expression. This is not unique to PfMCA1, other enzymes such as kinases are unable to be expressed heterologously unless their enzymatic activity had been attenuated (Kemble *et al*., 2006; Piserchio *et al*., 2009)

Despite the successful expression, expression levels of the non-catalytic domain were extremely low in ΔYCA1 yeast cells. In contrast, intense bands were observed in WT yeast cells. This discrepancy is unexpected, as there would be no plausible reason why the non-catalytic domain would be better expressed in WT cells.

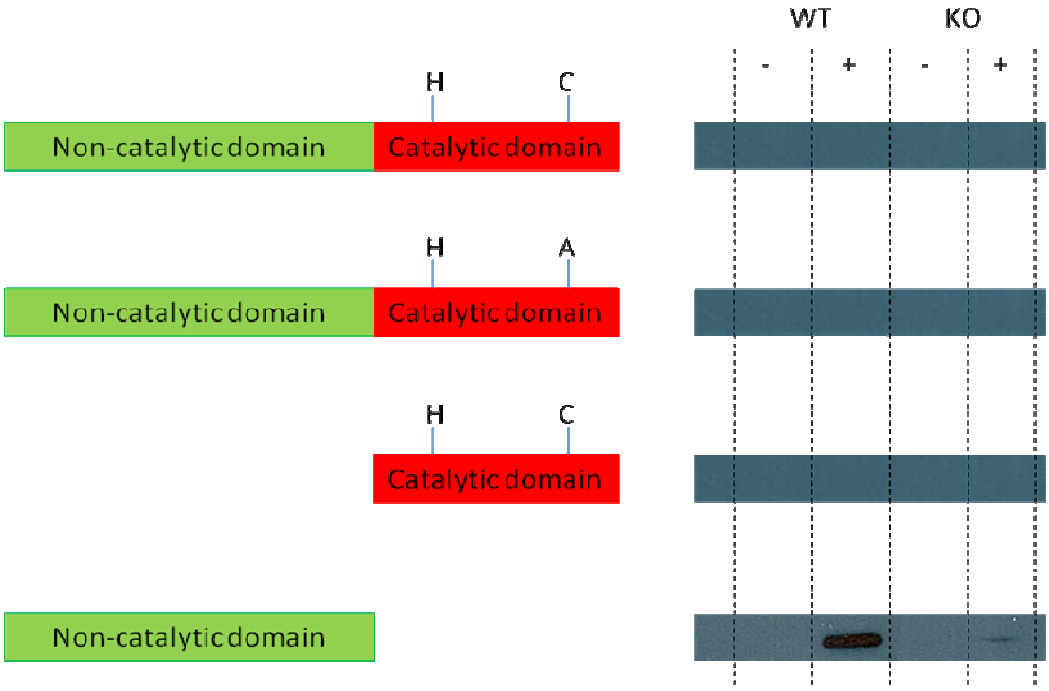

Figure 14. Schematic summary of PfMCA1 expression in *S. cerevisiae*.
The full-length PfMCA1 protein could not be expressed in yeast, and neither could a mutant with the critical cysteine residue replaced with an alanine. PfMCA1 can be broadly divided into non-catalytic and catalytic domains. While expression of the catalytic domain could not be detected, the non-catalytic domain could be expressed and detected in yeast cells under inducing conditions. Curiously, expression of the non catalytic domain was significantly lesser in ΔYCA1 yeast cells than WT cells.

4.8 Future strategies for successful PfMCA1 expression

While a variety of expression hosts have been examined in their ability to express PfMCA1, there still remain other alternatives. A promising alternative is the baculovirus system (Birkholtz *et al*., 2008). A significant advantage of the baculovirus expression system is that it recognizes various eukaryotic targeting and post-translational modification signals. Plasmodial proteins expressed in a baculovirus system are therefore likely to remain soluble and possess a native folding pattern. This approach has been successfully used to study the biochemical characteristics of plasmodial proteins (Chia *et al*., 2005; Rayavara *et al*., 2009) and for vaccine production (Lyon *et al*., 2008; Strauss *et al*., 2007; Yoshida *et al*., 2009).

Besides the baculovirus expression system, the slime mold *Dictyostelium discoideum* could also prove to be an attractive expression host for plasmodial proteins. It is relatively easy to culture and genetically manipulate (Birkholtz *et al*., 2008), and it also possesses caspase-like proteins called paracaspases (Uren *et al*., 2000). *D. discoideum* has already been used to study cell death pathways (Tresse *et al*., 2008), and the (A+T)-bias of its genome is similar to that of *P. falciparum* (Szafranski *et al*., 2005), making it more likely that any plasmodial proteins would be expressed without any significant problems. *D. discoideum* is therefore an attractive host system in which to functionally characterize plasmodial proteins. To date, several plasmodial proteins have been successfully expressed in *D. disoideum* (Fasel *et al*., 1992; Naudé *et al*., 2005; Sá *et al*., 2006; van Bemmelen *et al*., 2000).

Increased yield of PfMCA1 can also be achieved by fusing to it highly-stable proteins such as human γ-interferon and ubiquitin. This has been used successfully to obtain high yields of *P. falciparum* SERA proteins (Barr *et al*., 1991). While this could boost the amount of protein available for study, the fusion protein could lead to complications in biochemical analyses. It could also lead to immunogenicity problems, and plasmodial proteins produced in this manner are not used for therapeutic intervention (Bathurst, 1994).

A relatively new approach to the heterologous expression of plasmodial proteins, termed ‘codon harmonization’, takes into account the rate of protein translation in the parasite, and attempts to replicate the same rate in the expression host (Angov *et al*., 2008).

The rate of peptide elongation varies throughout the entire translation process, and at regions where translation slows down, partial folding of the protein can occur, increasing the stability of the nascent protein. Replacing these regions with high-abundance codons would prevent such stabilization, with the result that protein expression is significantly decreased. This approach has been used successfully to express plasmodial proteins which are potential vaccine candidates (Angov *et al*., 2008; Chowdhury *et al*., 2009).

Biochemical analysis of PfMCA1 had been hampered by the lack of protein expression, due to the perceived toxicity. It is possible to bypass any intracellular accumulation by attaching a secretory signal to PfMCA1. Any PfMCA1 produced would therefore be transported out of the cell into the extracellular medium via the secretory pathway, and can be purified. The secretory pathway minimizes any contact PfMCA1 might have with intracellular components, and thus increases the tolerance the cell might have for PfMCA1. This approach has been used to express a variety of proteins such as human serum albumin (Sleep *et al*., 1990), HIV proteins (Lasky *et al*., 1986) and cellulase complexes (Van Rensburg *et al*., 1998). The yeast α-factor mating pheromone is most commonly used for this purpose (Bathurst, 1994), and it involves the addition of the α-factor signal leader sequence to the N-terminus of the protein. The signal sequence is cleaved off before secretion, forming the mature protein with a correct N-terminus. Plasmodial proteins produced in this manner have been successfully used to illicit antibody responses (Barr *et al*., 1991; Gozar *et al*., 1998), for vaccine cocktails (Bathurst *et al*., 1993).

An alternative to the heterologous expression of PfMCA1 would be to knock out, or down-regulate, the expression of PfMCA1 in *P. falciparum* parasites themselves. Traditional approaches to create gene knockouts in *Plasmodium* parasites via homologous recombination events require a long period of time (typically 3 months), and this is further hampered by the low levels of efficiences in introducing the DNA into the parasites (Gardiner *et al*., 2003; Skinner-Adams *et al*., 2003). While this approach had been considered during the couse of this study (for understanding PfMCA1 functions), the impracticalites (a long period of time

required to generate successful mutants and unfamilarity with the techniques involved) precluded it from being put into use.

In recent years, transposon mutagenesis has been used to study functional analysis of the *Plasmodium* genome (Balu *et al*., 2005, 2009; Balu and Adams, 2006). Compared to traditional methods, *piggyBac* transposon-mediated mutagenesis is more efficient. Although this method randomly integrates into the genome, it is possible to obtain mutants with only one, single *piggyBac* insertion (Balu *et al*., 2009). It is then possible to screen for mutants of the gene of interest.

5. CONCLUSION

A literature survey only revealed one study that has managed to successfully express PfMCA1 (Meslin *et al*., 2007). Expressed PfMCA1 proteins appear to be processed in a similar fashion as caspases. However, the study did not characterize the functional aspects of PfMCA1, and its role in programmed cell death remains unknown.

Although PfMCA1 had been successfully cloned, it could not be expressed in *S. cerevisiae*, *E. coli*, and only at extremely low levels in *T. brucei*, despite our best efforts. Further investigation revealed that the most probable reason for this is the catalytic activity of PfMCA1. The non-catalytic domain of the protein could be successfully expressed, but the catalytic domain remained unexpressed. Suppression of the catalytic activity via site-directed mutagenesis did not prevent non-expression. However, only a clone where the cysteine residue of the catalytic dyad was mutated to alanine was generated. No clones in which the histidine residue or both the residues were mutated, were generated. The possibility remains that expression of the full-length PfMCA1 protein could be achieved if the catalytic activity was suppressed further, or even abolished altogether.

We have shown that the most reasonable explanation for the inability to express PfMCA1 in a variety of host organisms lies with the toxicity of its catalytic domain. Future directions for PfMCA1 would ideally expand on characterizing its catalytic function, and how it affects its own expression. The difficulties experienced in expressing this particular protein could explain why there is a dearth of information about it in current literature. PfMCA1-knockouts in *P. falciparum* would also complement any data obtained from function-complementation studies

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7. APPENDIX

7.1 PCR primers

Name	DNA Sequence
5'PfMCA-EcoRI	GCCGAATTCATGGAAAAAATATACGTCAAAAT
3'PfMCA-SalI	GGGCGTCGACTAAAAAAAAAATAAATTTTAAGTTC
5'YCA1-EcoRI	GCCGAATTCATGTATCCAGGTAGTGGAC
3'YCA1-SalI	GGGCGTCGACTACATAATAAATTGCAGATTTA
3'-PfMCA-6xHis-SalI	GGCGTCGACTAGTGATGATGGTGATGATGAAAAAAAAATAAATTTTTAAGTTC
3'-YCA1-6xHis-SalI	GCGTCGACTAGTGATGATGGTGATGATGCATAATAAATTGCAGATTTACG
ScActinFW	GGTTGCTGCTTTGGTTATTGA
ScActinRV	TGTGGTGAACGATAGATGGA
EcoRI-ScActin-fw	GCCGAATTCATGGAtTcTGaGGTT
NotI-ScActin-rv	TATAGCGGCCGCGAAACACTTGTGGTG
OpPfMCA-fw	GCCGAATTCATGCACCACCATC
OpPfMCA-noHis-fw	GCCGAATTCATGGAGAAAATTTATGTCAAG
OpPfMCA-pXS2-fw	GCCGCTAGCATGGAGAAAATTTATGTCAAG
OpPfMCA-rv	TATAGCGGCCGCGAAGAAAAATAAATTC
Gal10-fw	GGTGGTAATGCCATGTAATATG
Gal10-rv	GGCAAGGTAGACAAGCCGACAAC
PfActin-fw-1119	GCAGCAGGAATCCACACAAC
PfActin-rv-1402	GTGGACAATACTTGGTCCTG
OpPfMCA-C460A-fw	GCTGTTGTAGATTCGGCTAATAGCGGTTCTTC
OpPfMCA-C460A-rv	GAAGAACCGCTATTAGCCGAATCTACAACAGC
OpPfMCA-H404A-fw	CTATTTTCTGGTGCTGGCTCTCAGGAG
OpPfMCA-H404A-rv	CTCCTGAGAGCCAGCACCAGAAAATAG
pGEX-fw	GGGCTGGCAAGCCACGTTTGGTG
pGEX-rv	CCGGGAGCTGCATGTGTCAGAGG
OpPfMCA-cd-fw	GCCGAATTCAAGAAGGCATTACTGATCGG
OpPfMCA-cd-rv	TATAGCGGCCGCTTGCTTTACATGAGCATGG
YCA1-NotI-rv	TATAGCGGCCGCCATAATAAATTGCAGATTTA
FLAG-XhoI-rv	CTCGAGCTTATCGTCGTCATCCTTGTAATC
OpPfMCA-nonCD-rv	TATAGCGGCCGCTTGGTTACATGGACTGAAGTC
ScActinRTCtrl-fw	GACCAAACTACTTACAACTCCA
ScActinRTCtrl-rv	CATTCTTTCGGCAATACCTG
TbActin-fw	CAACGTGCTACTGACTGAGGCG
TbActin-rv	GCACTGTTCGTCATCTCTTCGTCG
TbMCA4-fw	GCTGCGTCAGTACTGCATTGAAAG
TbMCA4-rv	GTATTGTCAACGCCCAACGCTGC

Underlined bases represent restriction sites.

7.2 Sequencing primers

Name	DNA Sequence
PfMCA-Pact-5'-2295	CCTCACCCTAACATATTTTCCAATTAAC
PfMCA-Pact-5'-2700	CTTACTGCTTTTTTCTTCCCAAG
PfMCA-Pact-5'-3100	ATTGATGTTGTAAAGAAATGTACATTGC
PfMCA-Pact-5'-3500	ATAGCACTTATATGAACAATTCACCTAC
PfMCA-Pact-3'-3800	GTACAACCATTCAATTCATATTTGG
PfMCA-Pact-3'-4300	AAGAAACTTCCTTATCTTTACATCCAC
PfMCA-Pact-3'-4700	AGGGTGGTTTAAAAATAGAAATAGAG
PfMCA-Pact-3'-5025	AAAACGCCGGACTCAAATTCTAATG
YCA1-Pact-5'-2296	CTCACCCTAACATATTTTCCAATTAAC
YCA1-Pact-5'-2700	CTTACTGCTTTTTTCTTCCCAAG
YCA1-Pact-5'-3100*	GGTCCACCCCAGAATATGTCATTACCTC
YCA1-Pact-5'-3500**	TTATATATCCGGTCGATTTCGAAACTC
YCA1-Pact-3'-3350‡	ACCAAATCGTTCTGATCATCAG
YCA1-Pact-3'-3750‡‡	AGCAGCCCTGTTTCCTGTGGCATATG
YCA1-Pact-3'-4150	GTTTAAAAATAGAAATAGAGAGAGAGGTAC
YCA1-Pact-3'-4486	GTATCAAAACGCCGGACTCA
Pgal-5'	AAATCCACATAACTGACAAAACTGG
PfMCA-Pgal-5'-3740	CCAAATTATAGACCTACAAGAAGAAATA
YCA1-Pgal-5'-3740	GCTGTCGAAGATGGGCAAAATAC
PfMCA-pESC-fw-4098	GGAGAGTCTTCCTTCGGAGG
PfMCA-pESC-fw-4495	CATGTATCTTGCAGAAGAATCCATAC
PfMCA-pESC-fw-4893	ATTGGACAGTATAACAATATATACTTTAACG
PfMCA-pESC-fw-5301	CCGGGAAGTGATCAAACTTTATAC
PfMCA-pESC-rv-5202	GATTGGAGTTATGTAAATCATTAGATGC
PfMCA-pESC-rv-5601	GACCAGAAAATAGGAAGAACAGAATG
PfMCA-pESC-rv-6001	GTAATAATCGAAGGAGTGTTCATATTATTC
PfMCA-pESC-rv-6400	TATCTACCAACGATTTGACCCTTTTC
YCA1-pESC-fw-4243	CAACATATAAGTAAGATTAGATATGGATATG
YCA1-pESC-fw-4704*	GGTCCACCCCAGAATATGTCATTACCTC
YCA1-pESC-fw-5104**	TTATATATCCGGTCGATTTCGAAACTC
YCA1-pESC-rv-4933‡	ACCAAATCGTTCTGATCATCAG
YCA1-pESC-rv-5329‡‡	AGCAGCCCTGTTTCCTGTGGCATATG
YCA1-pESC-rv-5727	GATAAGATCTGAGCTCTTAATTAACAATTC

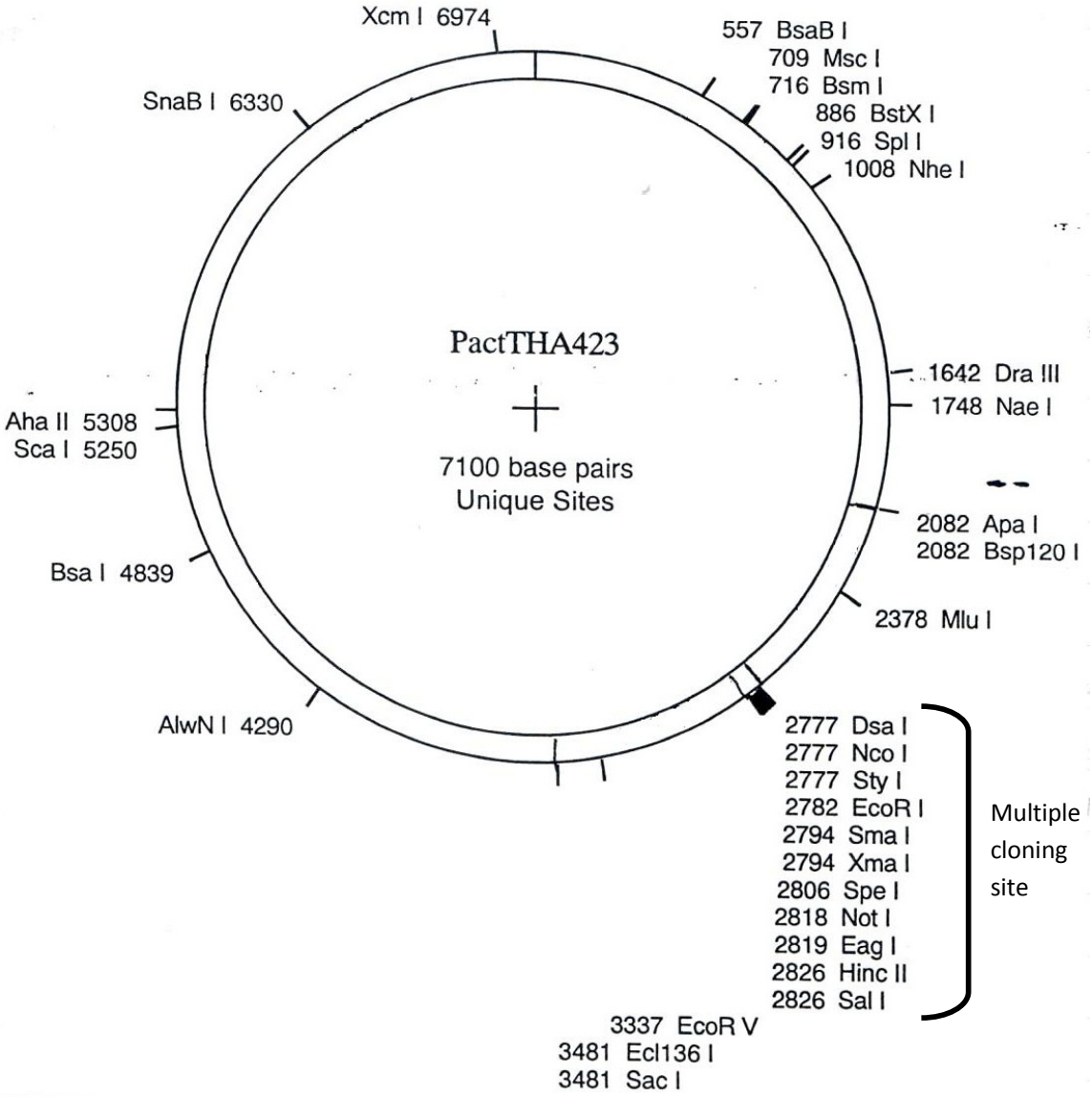
Sequences with the same symbol after their name have the same nucleotide sequences.

7.3 PactTHA423

PactTHA423 -> Graphic Map

DNA sequence 7100 b.p. TCGCGCGTTTCG ... GGCCCTTTCGTC circular

HIS3-marked 2um expression plasmid, carrying the actin1 promoter/ terminator cassette and an HA-tag.
Sites: ApaI-Pact1-HA-NcoI/EcoRI/SmaI/SpeI/NotI/HincII/SalI-Tact1-SacI

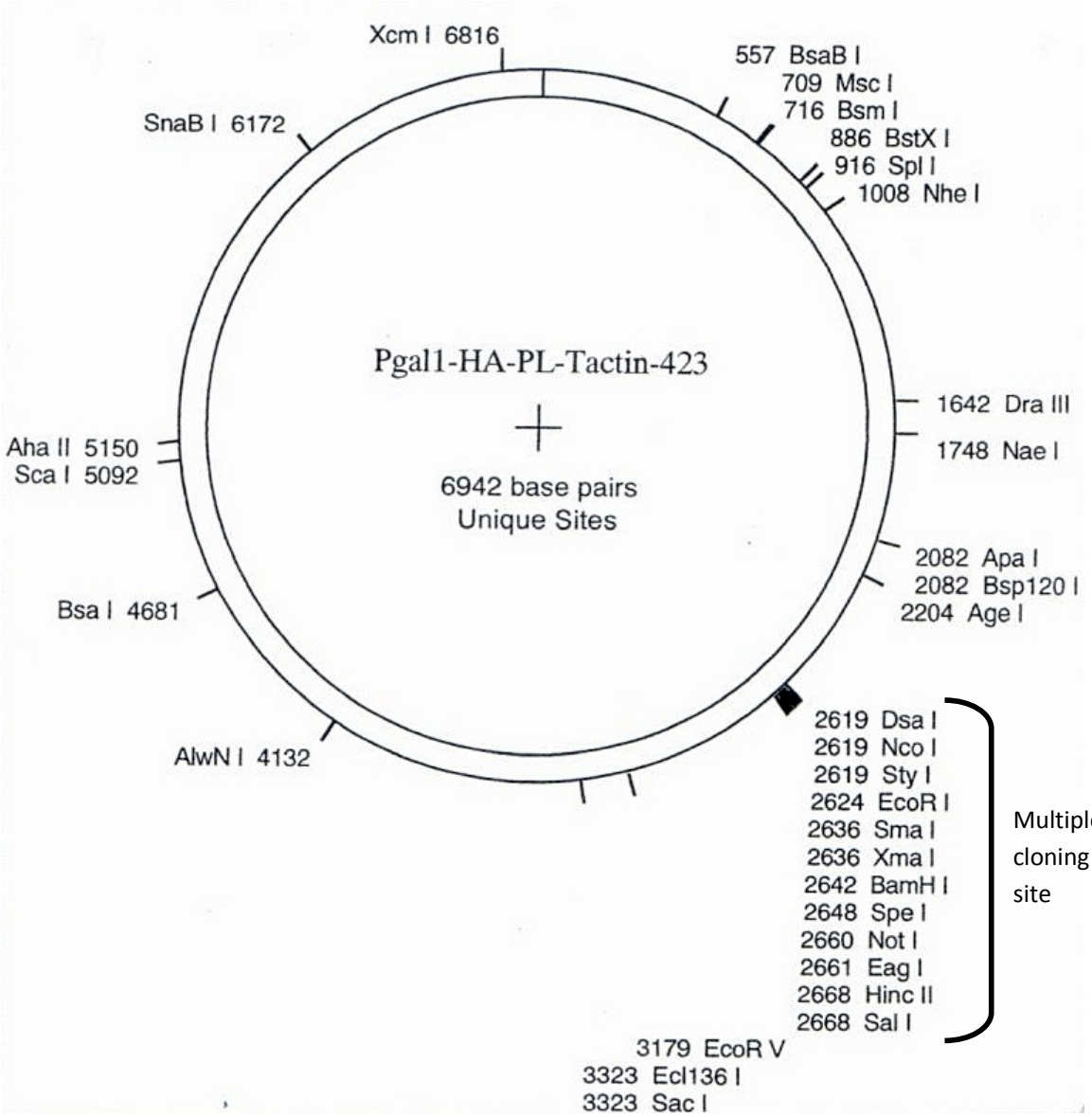


7.4 Pgal1-HA-PL-Tactin-423

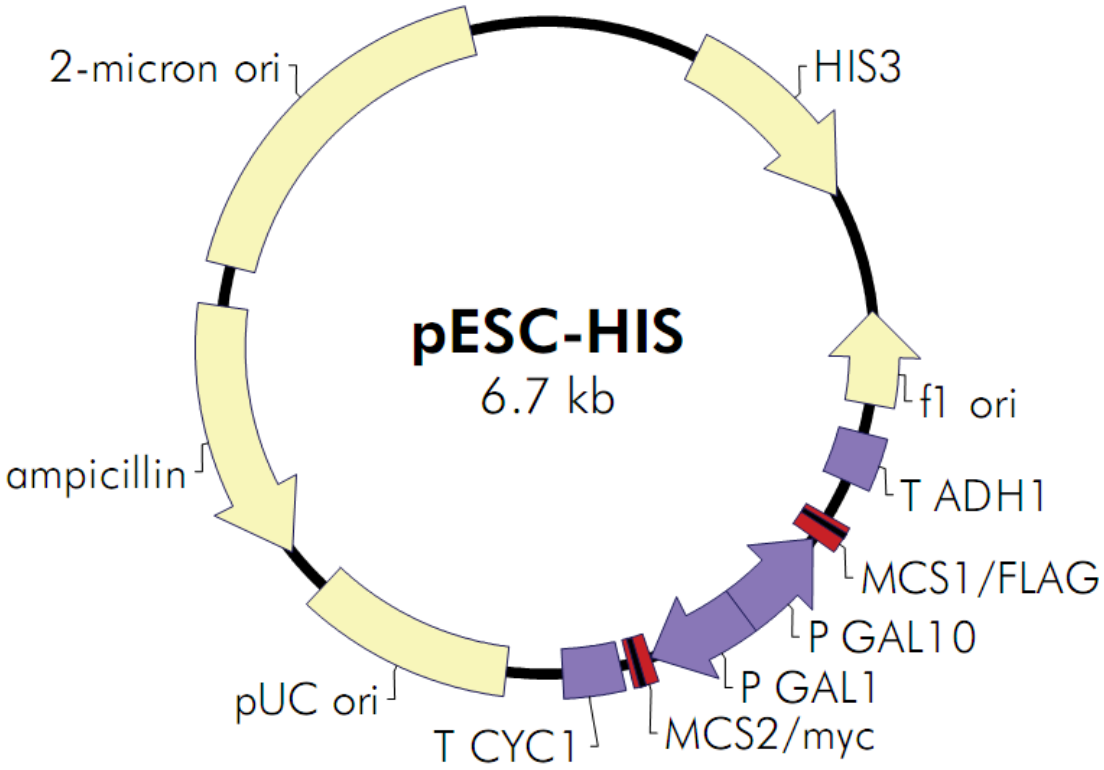
Pgal1-HA-PL-Tactin-423 -> Graphic Map

DNA sequence 6942 b.p. TCGCGCGTTTCG ... GGCCCTTTCGTC circular

2um, HIS3-marked expression plasmid, carrying an GAL1 promoter/actin terminator cassette and an HA-tag.
Sites: ApaI-Pgal1-HA-NcoI/EcoRI/SmaI/ SpeI/NotI/HincII/SalI-Tact1-SacI



7.5 pESC-HIS



pESC-HIS Multiple Cloning Site 1 Region
(sequence shown 2255–2338, bottom strand)

EcoR I | Not I | Spe I | Cla I | Sac I | Pac I

GAA TTC AAC CCT CAC TAA AGG GCG GCC GCA CTA GTA TCG ATG GAT TAC AAG GAT GAC GAC GAT AAG ATC TGA GCTCTTAATTAA

M D Y K D D D D K I STOP

FLAG epitope

pESC-HIS Multiple Cloning Site 2 Region
(sequence shown 3011–3108, top strand)

BamH I | Apa I | Srf I | Sal I

G GAT CCG TAA TAC GAC TCA CTA TAG GGC CCG GGC GTC GAC...

(STOP)

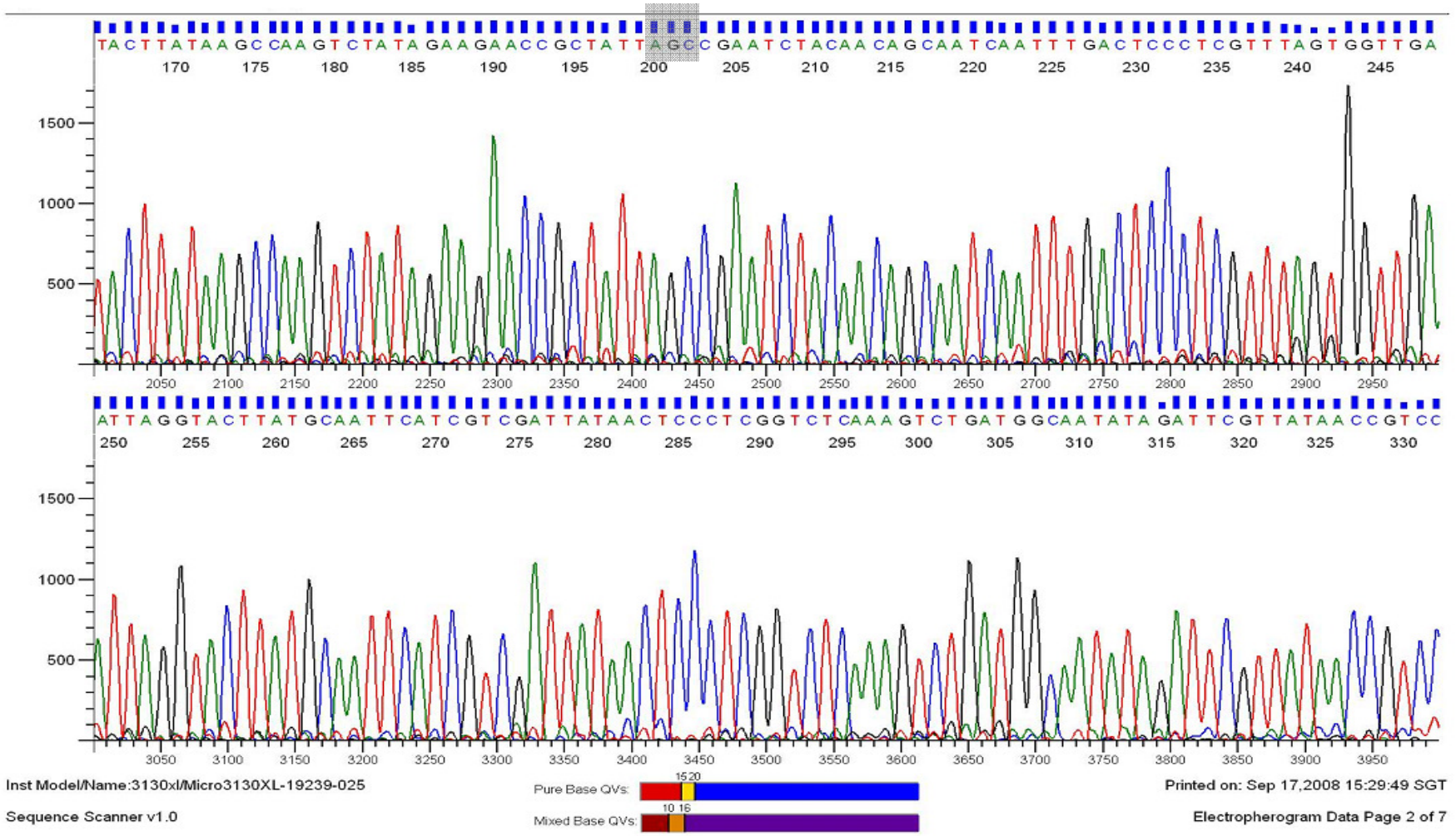
Xho I

...ATG GAA CAG AAG TTG ATT TCC GAA GAA GAC CTC GAG TAA GCTTGGTACCGCGGCTAGC

M E Q K L I S E E D L E STOP

myc epitope

7.6 Electropherogram of PfMCA C460A mutant



Electropherogram of PfMCA1 with a C460A mutation. The sequencing was done in the 3'→5' direction, hence the results are a reverse-complement of the coding sequence. The mutation is boxed in grey

7.7 Data from ConA assay

[ConA] = 1.0 μg/ml

Raw Data								Cell Count			
Time	[ConA] (μg/ml)	Sample	Time (hr)	Tet-		Tet+		Tet-		Tet+	
				ConA-	ConA+	ConA-	ConA+	ConA-	ConA+	ConA-	ConA+
24	1.0	1	24	4	4	9	6	2000000	2000000	4500000	3000000
48			48	24	6	24	12	12000000	3000000	12000000	6000000
72			72	42	17	41	29	21000000	8500000	20500000	14500000
96			96	26	16	25	26	13000000	8000000	12500000	13000000
24		2	24	15	12	7	7	7500000	6000000	3500000	3500000
48			48	28	15	29	13	14000000	7500000	14500000	6500000
72			72	30	29	64	27	15000000	14500000	32000000	13500000
96			96	24	32	33	33	12000000	16000000	16500000	16500000

Average Cell Count						Standard Error			
Time	[ConA] (μg/ml)	Tet-		Tet+		Tet-		Tet+	
		ConA-	ConA+	ConA-	ConA+	ConA-	ConA+	ConA-	ConA+
24	1.0	4750000	4000000	4000000	3250000	2750000	2000000	500000	250000
48		13000000	5250000	13250000	6250000	1000000	2250000	1250000	250000
72		18000000	11500000	26250000	14000000	3000000	3000000	5750000	500000
96		12500000	12000000	14500000	14750000	500000	4000000	2000000	1750000

[ConA] = 2.5 μg/ml

Raw Data							Cell Count			
Time (hr)	[ConA] (μg/ml)	Sample	Tet-		Tet+		Tet-		Tet+	
			ConA-	ConA+	ConA-	ConA+	ConA-	ConA+	ConA-	ConA+
24	2.5	1	15	2	12	0	7500000	1000000	6000000	0
48			46	5	29	1	23000000	2500000	14500000	500000
72			28	9	47	12	14000000	4500000	23500000	6000000
96			14	6	21	8	7000000	3000000	10500000	4000000
24		2	11	1	15	11	5500000	500000	7500000	5500000
48			30	0	40	12	15000000	0	20000000	6000000
72			48	12	30	10	24000000	6000000	15000000	5000000
96			20	6	26	13	10000000	3000000	13000000	6500000
24		3	17	3	17	1	8500000	1500000	8500000	500000
48			66	4	43	8	33000000	2000000	21500000	4000000
72			42	7	41	13	21000000	3500000	20500000	6500000
96			23	7	27	11	11500000	3500000	13500000	5500000

Average Cell Count						Standard Error			
Time	[ConA] (µg/ml)	Tet-		Tet+		Tet-		Tet+	
		ConA-	ConA+	ConA-	ConA+	ConA-	ConA+	ConA-	ConA+
24	2.5	7166666.7	1000000	7333333	2000000	881917.1	288675.1	726483.2	1755942
48		23666667	1500000	18666667	3500000	5206833	763762.6	2127858	1607275
72		19666667	4666667	19666667	5833333	2962731	726483.2	2488864	440958.6
96		9500000	3166667	12333333	5333333	1322876	166666.7	927960.7	726483.2

Student's t-test (unpaired, two-tailed, unequal variance)

[ConA] (μg/ml)	Time	Sample	Tet-		Tet+	
			ConA-	ConA+	Con-	ConA+
1.0	24	1	2000000	2000000	4500000	3000000
		2	7500000	6000000	3500000	3500000
	p-value		0.4237996		0.1749428	
	48	1	12000000	3000000	12000000	6000000
		2	14000000	7500000	14500000	6500000
	p-value		0.0689624		0.050831	
	72	1	21000000	8500000	20500000	14500000
		2	15000000	14500000	32000000	13500000
	p-value		0.1325983		0.1386873	
	96	1	13000000	8000000	12500000	13000000
		2	12000000	16000000	16500000	16500000
	p-value		0.4604844		0.4668806	

[ConA] (µg/ml)	Time	Sample	Tet-	Tet+	Tet-	Tet+
			ConA-	ConA+	ConA-	ConA+
2.5	24	1	7500000	1000000	6000000	0
		2	5500000	500000	7500000	5500000
		3	8500000	1500000	8500000	500000
	p-value		0.0065394		0.0386569	
	48	1	23000000	2500000	14500000	500000
		2	15000000	0	20000000	6000000
		3	33000000	2000000	21500000	4000000
	p-value		0.0241525		0.0029265	
	72	1	14000000	4500000	23500000	6000000
		2	24000000	6000000	15000000	5000000
		3	21000000	3500000	20500000	6500000
	p-value		0.0154381		0.0138861	
	96	1	7000000	3000000	10500000	4000000
		2	10000000	3000000	13000000	6500000
		3	11500000	3500000	13500000	5500000
	p-value		0.0195548		0.0024009	